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NEUROSECRETORY ACTIVITY IN RATS UNDER CONDITIONS OF CONTINUOUS LIGHT OR DARKNESS

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ABSTRACT

Cytological study of the posterior pituitary and hypothalamic areas of male and female rats kept under usual laboratory conditions (controls), in continuous light, or in constant darkness for eight weeks revealed differences in neurosecretory activity. While the paraventricular nuclei seemed to be unaffected by the varied light treatment, the cells of the supraoptic nuclei were largest and appeared to most active in the lighted rats, smallest and least active in the rats housed in darkness. The amount of neurosecretory material in the hypothalamo-hypophysial tract in two areas, namely, in the region ventral to the supraoptic nuclei and in the median eminence, was estimated from serial sections. It was apparent that Gomori-positive substance was most abundant and occurred in larger clumps along the hypothalamo-hypophysial tract in the continuously lighted rats. It was least in amount in the rats kept in darkness. The neurosecretory material in the posterior lobe was plentiful in the controls, but was reduced in both the other groups. The Herring bodies in the lighted animals were very small.

In light-treated rats large amounts of neurosecretory material appeared to be moving along the neurosecretory axones, while the pars nervosa of such animals was relatively depleted of Gomori-positive substance. This suggests that under the influence of added lighting, the rat both secretes and releases increased amounts of neurosecretory material.

At least a partial separation in function between the paraventricular nuclei and the supraoptic nuclei was indicated by the difference in response of these two nuclei.

PREVIOUS work has shown that light is an important extrinsic factor in the regulation of reproductive activity in many vertebrates. There is also considerable evidence that added lighting increases the secretion and release of the follicle stimulating hormone (FSH) of the anterior pituitary in the rat. Continued exposure to light induces precocious sexual

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maturity, followed in the female by periods of persistent estrus (1, 2, 3). Study of several mammals indicates that the central nervous system plays a role in these phenomena, since an intact retina and optic nerve are essential in the transmission of the light stimulus (4). More recently evidence has been presented which indicates that a neurosecretory link between the anterior pituitary and the hypothalamus is also involved (5). The possibility that the neurosecretory cells of the supraoptic and paraventricular nuclei are the source of such a neurosecretion is supported by anatomical studies showing that axones from the neurosecretory cells constituting these nuclei are closely associated in the median eminence with capillaries which drain into portal vessels supplying the anterior pituitary (6, 7). In addition, work on stalk-sectioned animals (8, 9) and with pituitary grafts (10) shows that a vascular connection between the median eminence and the anterior hypophysis is necessary for the maintenance of cyclic reproductive activity.

In view of the possible importance of the supraoptic nuclei and the paraventricular nuclei in governing some normal secretory functions of the anterior pituitary, a study of their cytology as well as that of the hypothalmo-hypophysial tract in rats housed under conditions of artificially regulated illumination was undertaken.

METHODS

Twenty-two-day old male and female rats were placed in cages and a) exposed to constant illumination from 100w bulbs, b) kept under usual laboratory conditions (12 hours of light, 12 hours of darkness), or c) housed in nearly complete darkness. Eight weeks later the animals were killed instantly, organ and body weights were recorded, and the ovaries were inspected for gross structural features before fixation. The pituitary glands and the hypothalamic area of the brains were fixed in Bouin's fluid. The hypothalamic area was sectioned serially at 10 μ and all sections were mounted. The sections were taken in the frontal plane in fourteen rats and in the sagittal plane in six. All the pituitaries were sectioned at 3μ in the horizontal plane. Three staining methods were employed: 1) Gomori's aldehyde fuchsin (after 1 minute's oxidation with 0.3% potassium permanganate solution and bleaching with 2.5% sodium bisulfite), 2) Gomori's aldehyde fuchsin followed by Halmi's counter-stain (11)[2], and 3) Gomori's chrome-alum-hematoxylin and phloxine (12). Aldehyde fuchsin and chrome-alum-hematoxylin make possible the visualization of neurosecretory material and/or its carrier (6). The dyes used vary in staining capacity with age and temperature, hence only slides stained simultaneously were compared.

Vaginal smears of continuously lighted rats and those housed under regular laboratory conditions were taken daily for two-and-a-half weeks prior to necropsy.

A study of the cytological changes found in the pars distalis in these animals will be reported later.

RESULTS

The hypothalamus in 20 male animals (7 controls, 7 kept in darkness, 6 maintained under constant light) was examined. No difference in the ap-

[2] The concentration of light green used was .4% rather than .2%.

pearance or apparent secretory activity of the cells of the paraventricular nuclei was detected when the three groups of animals were compared. In every instance many of these cells were well filled with neurosecretory granules, and substantial amounts of neurosecretory material were consistently present in the axones leading away from the nuclei. In contrasting fashion the cytology of the supraoptic nuclei varied with the exposure to light. In the control rats the cells of these nuclei were found to be much like those of the paraventricular nuclei except for the more frequent appearance of small, often spindle-shaped cells with small nuclei and containing in the cytoplasm very coarse Gomori-positive granules having the appearance of hyaline material. These cells gave an impression of secretory inactivity. Of particular interest was the difference in the frequency with which these "inactive cells" occurred in each of the three groups. They were few in number in all the lighted rats, intermediate in incidence in the control group and abundant in 6 of the 7 rats housed in darkness. Cells of large size, displaying the characteristics of intense secretory activity, were most evident in the lighted group and sparsely present in the group housed in darkness (Figs. 1, 2). Their abundant cytoplasm contained in the periphery many fine neurosecretory granules, while clustered about the nuclei were coarser granules of similar staining properties. The nuclei were vesicular, with a conspicuous nucleolus.

The amount of neurosecretory material passing from the hypothalamic nuclei to the pars nervosa was estimated in serial sections. The estimation was made difficult by the fact that the neurosecretory content detectable at different levels varied considerably. Moreover, the neurosecretory products emanating from different hypothalamic nuclei cannot be distinguished in the tractus hypophysius, since axones from the paraventricular nuclei run along beside those of the supraoptic nuclei through much of this area. Mindful of these conditions and limitations, we made comparisons of the neurosecretory material found in the hypothalamo-hypophysial tract at two points; namely, just ventral to the supraoptic nuclei (Figs. 3, 4, 5) and in the median eminence (Figs. 6, 7, 8). Though the distinctions were not always sharp, it was evident, as the illustrations faithfully depict, that at both levels the greatest amount of secretion was found in lighted rats, the least in those kept in darkness. The character of the neurosecretory material varied, too, in that heavy accumulations were commonly found in light-stimulated animals, while slender strands of neurosecretory material were typical of rats deprived of light.

It is, of course, not possible to judge by these means alone whether the neurosecretion passes into the capillaries of the median eminence and, if so, to what degree. It was, however, of great interest to note that the neurosecretory droplets became increasingly small, with increasing proximity to the blood vessels (shown best in Fig. 6). Beyond this point they could not be identified in these preparations with any degree of accuracy.

Study of the pars nervosa of 46 rats, both male and female (10 kept in darkness, 17 under continual lighting, and 19 under usual laboratory conditions) revealed differences in the amount of Gomori-positive material present. In animals living under normal laboratory conditions the neurosecretory material was most abundant, whereas in rats maintained under extreme excess or absence of light its amount was notably lessened (Figs. 9, 10, 11). Neurosecretory droplets were prominent in Herring bodies and along the axones in the control animals, while in the lighted rats these were scanty in both these sites. The decrease in neurosecretory material observed in the rats kept in darkness was largely confined to the axones, the Herring bodies usually being moderately large. It must be emphasized that within all three groups there was variation in response, yet when one group was compared with another, the changes noted were clearly evident. In Table 1 we have recorded our subjective estimates of the relative abundance of neurosecretory material in axones and Herring bodies on the basis of a scale of + to +++.

Examination of the vaginal smears revealed that while the rats housed

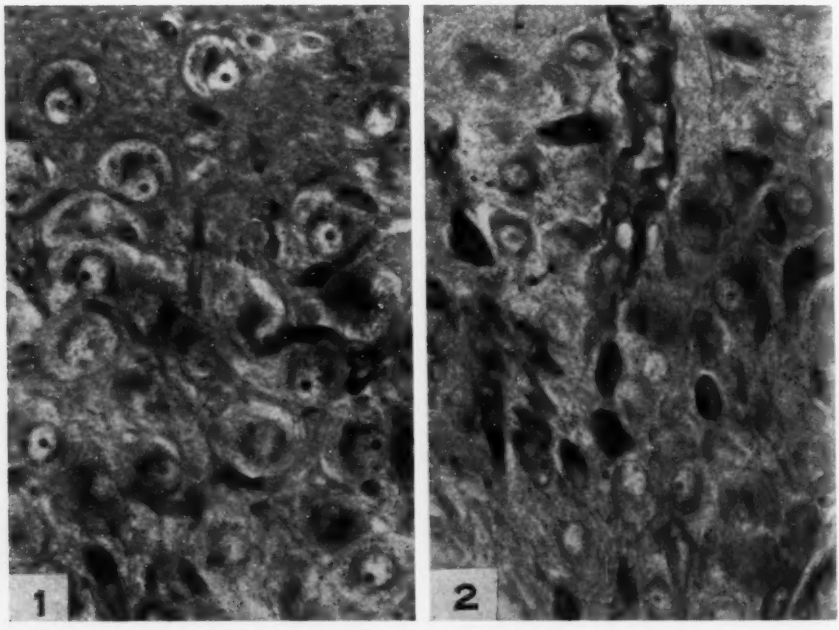
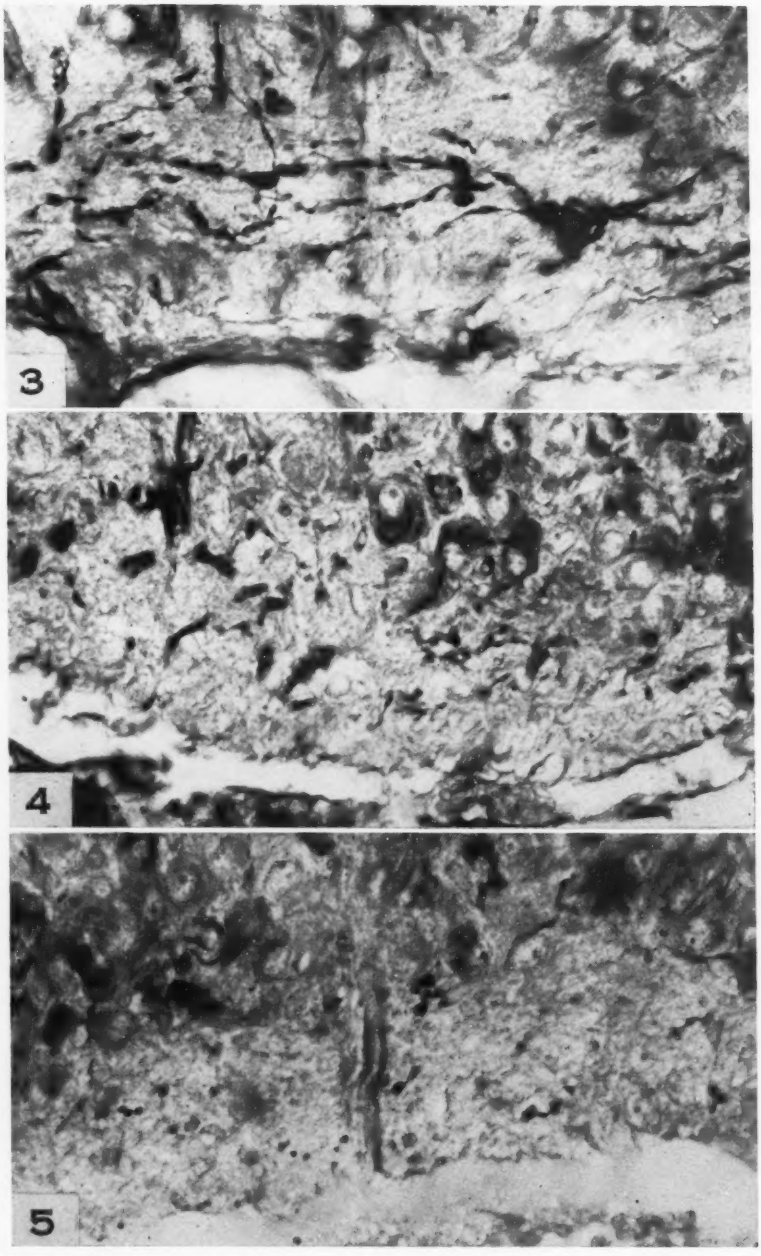
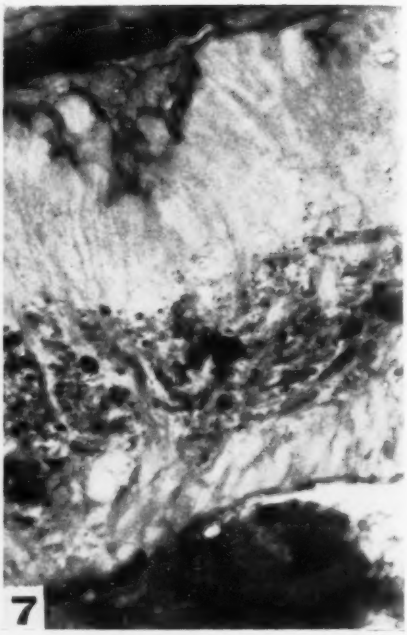


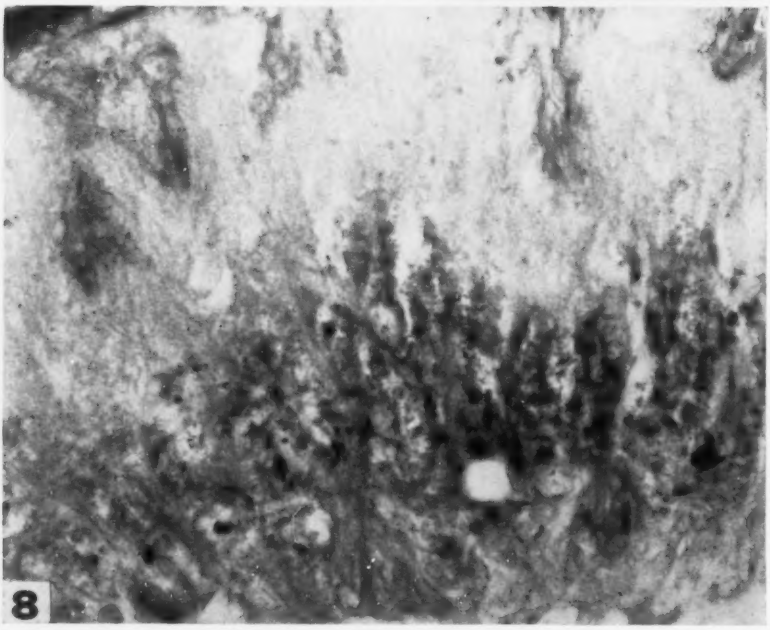
FIG. 1. Section through the supraoptic nucleus in a male rat continuously exposed to light showing the large size and active appearance of the neurosecretory cells characteristic of such animals. Aldehyde fuchsin and Halmi's counterstain. ×500. 10μ.

FIG. 2. Section through a supraoptic nucleus in a male rat kept in darkness showing some spindle cells and other neurosecretory cells typical of such animals. Aldehyde fuchsin and Halmi's counterstain. ×500. 10 μ.



FIGS. 3–5. Sections through the hypothalamo-hypophysial tract ventral to the supraoptic nucleus in male rats kept in the light, under usual laboratory conditions and in darkness respectively. Aldehyde fuchsin and Halmi's counterstain. ×500. 10μ.





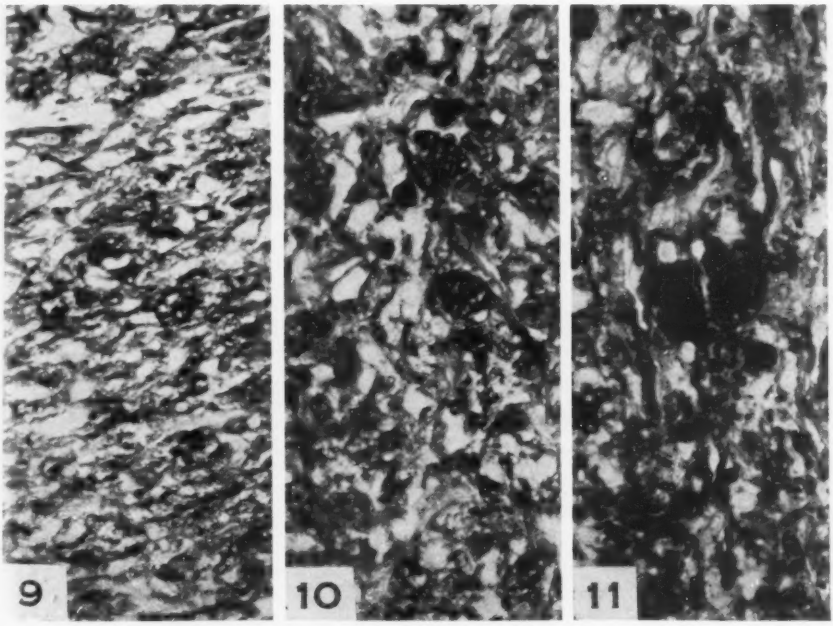
FIGS. 6–8. Sections through the hypothalamo-hypophysial tract in the median eminence in male rats kept in light, under usual laboratory conditions and in darkness respectively. Note the axones and associated Gomori positive material near the capillaries in Fig. 6. Aldehyde fuchsin and Halmi's counterstain. ×500. 10 μ.

under usual laboratory conditions were cycling regularly (showing the 4-day cycle typical of this strain), those in continuous light were cycling at intervals of approximately 7 days due to lengthened periods of estrus. Smears were not taken on the animals housed in darkness, but one of us has previously reported (3) that under similar conditions such rats cycle irregularly, exhibiting many metestrus smears.

Organ and body weights are presented in Table 2 and are in agreement with previous work in reflecting the gonad-stimulating action of added lighting. The appearance of the ovaries, too, was in line with earlier findings. Those of continuously lighted females, though not devoid of corpora lutea, had conspicuously large follicles, whereas the ovaries of the females housed in darkness were generally small, containing few well developed corpora lutea or follicles.

DISCUSSION

The observations reported here support the view that a neurosecretory link may be involved in the stimulation of the pituitary by light. Of especial interest is the fact that the supraoptic nuclei appear to be affected by changes in optic stimulation, while the paraventricular nuclei are not. These two pair of nuclei are represented in the amphibia by the single N



FIGS. 9, 10 and 11. Sections through the pars nervosa of male rats exposed to altered light conditions for eight weeks. (9) Rats in continous light, (10) in constant darkness, (11) under control conditions. Note the differences in the amount of neurosecretory material in the Herring bodies and along the axones. Aldehyde fuchsin. ×600. 3μ.

TABLE 1. RELATIVE AMOUNTS OF NEUROSECRETORY MATERIAL IN AXONES AND HERRING BODIES THROUGHOUT THE NEUROHYPOPHYSIS*

Control Rats			Rats in Constant Light			Rats in Constant Darkness		
	Axones	Herring bodies		Axones	Herring bodies		Axones	Herring bodies
			Females					
1	++	++	1	+	+	1	+	+
2	+++	+++	2	+	+	2	+	++
3	+++	+++	3	+	+	3	+	++
4	+++	+++	4	+	+	4	+	++
5	+++	+++	5	+	+			
6	+++	+++	6	+	+			
7	+++	+++	7	+	+			
8	+++	+++	8	++	+			
9	+++	+++	9	+	++			
10	++	++						
			Males					
1	+++	+++	1	+	+	1	++	++
2	+++	+++	2	+	+	2	+	+
3	+++	+++	3	+	++	3	+	+
4	+++	+++	4	+	+	4	+	+
5	++	++	5	+	+	5	+	++
6	+++	+++	6	+	+	6	++	++
7	++	++	7	+	+			
8	++	++	8	+	+			
9	++	+++						
19			17			10		Totals

* + Neurosecretory material scarce.
++ Neurosecretory material moderate.
+++ Neurosecretory material abundant.

praeopticus and both seem to be important in the production of posterior lobe hormones. It has been rather generally assumed that these nuclei perform common functions and respond similarly to experimental procedures. However, such a view is not consistent with the findings of Castor *et al.* (13) or with the extensive work of Olivecrona (14), whose studies point to the paraventricular nuclei as the primary source of oxytocin, and to the supraoptic nuclei as possibly the main source of vasopressin. Our

TABLE 2. BODY AND ORGAN WEIGHTS OF YOUNG MALE AND FEMALE RATS KEPT IN CONSTANT LIGHT, TOTAL DARKNESS, OR UNDER NORMAL CONDITIONS FOR EIGHT WEEKS

Treatment	No. of animals	Body wt. avg., gm.	Ovary avg. wt., mg.	Uterus avg. wt., mg.	Adrenal wt. avg., mg.
Light	15	184.8	68.4	342	47.7
Control	13	189.7	64.9	300	46.9
Dark	10	181.0	41.3	190	42.8
			Testes avg. wt., gm.	Sem. ves. avg. wt., mg.	
Light	18	223	2.96	375	30.8
Control	11	227	2.61	266	34.6
Dark	10	256	2.76	280	31.1

data likewise suggest some dissimilarity in the function of the supraoptic and paraventricular nuclei.

It is not possible on the basis of the present study either to draw conclusions concerning the mode of action of light on the supraoptic nuclei or to evaluate the functional significance, if any, of the morphological changes noted. The increased neurosecretory activity may have been induced directly by continuous optic stimulation in which event it may be related to the well-known light-induced changes in anterior pituitary secretion. The most evident shift in the anterior pituitary hormone balance brought about by constant lighting is the increased secretion and release of FSH and a tendency for the secretion of LH to be suppressed (3). The thyroids and adrenals of the light-exposed rats used in this study appeared to be functioning at normal levels as judged by weight and cytological appearance. But, it is noteworthy that rats subjected to sudden intense light respond by releasing an increased amount of ACTH (15). There is also evidence that under the influence of continuous light TSH-release is reduced in some rodents (16, 17). Thus, under appropriate conditions, the secretion of four of the anterior lobe hormones (FSH, LH, TSH, and ACTH) seems to be affected by light. It remains to be determined whether changes in the neurosecretory activity of higher centers, as noted here, can be related to alterations in anterior lobe functions.

The abundance of neurosecretory substance in the supraoptic nuclei, combined with the relative paucity of such material in the neural lobe in the rats exposed to light, suggests an increased release of neurohypophysial hormones. These circumstances, coupled with concomitant changes in gonadal activity, point again to the possibility that posterior lobe hormones may play a role in anterior pituitary physiology. Mirsky *et al.* (18), McCann and Brobeck (19), Martini and Morpurgo (20), and McCann (21) have advanced evidence in support of the view that neurohypophysial hormones are involved in ACTH secretion. The evidence, reviewed by Sayers, Redgate and Royce (22), is as yet inconclusive. Recently, Benson and Folley (23) have found that oxytocin injected into lactating rats increases LTH secretion, and Desclin (24) and Stutinsky (25) have induced decidua in animals injected with oxytocin. At the present time, however, there is no clear explanation of the role, if any, of the neurohypophysis in the regulation of the anterior pituitary.

There is the further possibility that the increased activity of the supraoptic nuclei of the rats housed in light may be an indirect response. Cavallera *et al.* (26) have reported that estrogen has a diuretic action in the rat, and De Groot (27) has suggested that the increased neurosecretory material which he found in the pars nervosa of some of his spayed rats may have been caused by the absence of this action of estrogen. Any diuretic action which estrogen may have had in the rats exposed to light as reported here was limited, for in no instance did the depletion of neuro-

secretory material approach that which follows severe dehydration as described by Leveque and Scharrer (28). The further observation by De Groot that some of his dehydrated rats continued to cycle although their posterior pituitaries were depleted of neurosecretory material is of interest. It suggests that cyclic reproductive activity can occur in the virtual absence of Gomori-positive material in the pars nervosa.

The "inactive" neurosecretory cells found in the rats housed in darkness were so classified in part because of their cytological features, but also because Hild and Zetler (29) have shown that neurosecretory cell bodies become filled with Gomori-positive material only when the cells have filled their "storage depots."

In view of the work reported here, assays of the posterior pituitary of light- and dark-treated animals might prove of interest. Furthermore, since previous work has shown that changes in anterior pituitary structure (30) and gonadotrophic content (3) are particularly marked in rats subjected to altered light conditions for the period used in this experiment, cytological studies of both the hypothalamus and the anterior pituitary in animals exposed for graded periods of time might be rewarding.

Acknowledgments

The authors are most grateful to Miss Anna Morse for her valuable suggestions and to Miss Nancy Cuteliffe for her technical assistance.

ADDENDUM

Since the submission of this paper, Oksche *et al.* have reported changes in neurosecretion in the sparrow in response to light. (*Anat. Rec.* **130:** 433 abst. 1958).

REFERENCES

1. Hemmingsen, A. M. and N. B. Krarup: *Det. Kgl. Danske Viderskabernes Selskab* **13:** 5. 1937.
2. Browman, L. G.: J. Exper. Zool. **75:** 375. 1937
3. Fiske, V. M.: *Endocrinology* **29:** 187. 1941.
4. Thomson, A. P. D.: *J. Physiol.* **113:** 425. 1951.
5. Benoit, J. and I. Assenmacher: *J. Physiologie* **47:** 427. 1955.
6. Scharrer, E. and B. Scharrer: *Rec. Prog. Horm. Res.* **10:** 183. 1954.
7. Dawson, A. B.: *Anat. Rec.* **128:** 77. 1957.
8. Harris, G. W.: *J. Physiol.* **111:** 347. 1950.
9. Greep, R. O. and R. J. Barrnett: *Endocrinology* **49:** 172. 1951.
10. Nikitovitch, M. B. and J. W. Everett: *Fed. Proc.* **16:** 94. 1957.
11. Dawson, A. B.: *Anat. Rec.* **115:** 63. 1953.
12. Gomori, G.: *Am. J. Path.* **17:** 395. 1941.
13. Castor, W. C., B. L. Baker, D. J. Ingle and C. H. Li: *Proc. Soc. Exper. Biol. & Med.* **76:** 353. 1951.
14. Olivecrona, H.: *Acta Physiol.* **40** (Suppl.): 136. 1957.
15. Fortier, C.: *Rev. Canad. Biol.* **9:** 70. 1950.
16. Dempsey, E. W. and H. F. Searles: *Endocrinology* **32:** 119. 1943.

17. Puntriano, G. and J. Meites: *J. Endocrinol.* **48:** 217. 1951.
18. Mirsky, I. A., M. Stein and G. Paulish: *Endocrinology* **55:** 28. 1954.
19. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exper. Biol. & Med.* **87:** 318. 1954.
20. Martini, L. and C. Morpurgo: *Nature* **175:** 1127. 1955.
21. McCann, S. M.: *Endocrinology* **60:** 664. 1957.
22. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **20:** 243. 1958.
23. Benson, G. K. and S. J. Folley: *J. Endocrinol.* **16:** 189. 1957.
24. Desclin, L.: *C. rend. Soc. biol.* **150:** 1489. 1956.
25. Stutinsky, F.: *C. rend. Acad. sc.* **244:** 1537. 1957.
26. Cavallera, C., S. Corbetta and B. Malandra: *Arch. internat. Pharmacodyn.* **87:** 366. 1951.
27. De Groot, J.: *Anat. Rec.* **127:** 201. 1957.
28. Leveque, T. F. and E. Scharrer: *Endocrinology* **52:** 436. 1953.
29. Hild, W. and G. Zetler: *Pflüger's Arch. ges. Physiol.* **257:** 169. 1953.
30. Pomerat, G. R.: *Anat. Rec.* **82:** 531. 1942.

OBSERVATIONS ON THE PRODUCTION OF GOITER IN RATS WITH PROPYLTHIOURACIL AND ON GOITER PREVENTION[1]

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ABSTRACT

Rats fed propylthiouracil and injected with sodium-*l*-thyroxine (1.0 to 2.5 μg. per rat per day) developed goiter in the presence of normal concentrations of plasma protein-bound iodine. At all dose levels of thyroxine, rats fed low residue diets had greater serum bound iodine concentrations than animals fed commercial laboratory chow. Since goiter was found in presence of normal or high bound iodine concentrations, this may mean (a) goiter can develop despite low plasma levels of thyrotropin under these conditions or (b) plasma protein-bound iodine may have an abnormal composition or influence in these types of experiments. A method is presented for determination of plasma protein-bound iodine by isotope dilution.

DEMPSEY and Astwood (1) introduced the goiter-prevention method for estimating the daily rate of thyroid hormone secretion and presented as reasonable the statement: "The amount of thyroxine necessary to maintain normal thyroid weight in thiouracil-treated animals is a valid index of the rate of secretion of thyroid hormone." This has been widely accepted. They also stated: "In this instance, the balance is considered to be between the degree of pituitary inhibition exerted by the thyroid hormone and the quantity of thyroid secretion under the stimulus of thyrotropic hormone. According to this concept, then, an index of the degree of thyroid stimulation is provided by the size of the gland, which may be expected to vary directly with the rate of secretion of the thyrotropic hormone and inversely with the concentration of the circulating thyroid hormone." Since that time the estimation of circulating thyroid hormone, as protein-bound iodine, has become simpler, although the quantitation of thyrotropin in plasma remains difficult. However, the assumptions of Dempsey and Astwood have appeared so altogether reasonable that meas-

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irement has been neglected even in regard to "the concentration of the irculating thyroid hormone."

In the course of studies on the effect of high and low residue diets on the ate of fecal excretion of thyroxine we were surprised to gather data indiating that rats fed propylthiouracil and injected with sodium-*l*-thyroxine n graded dosage developed goiters unless the plasma protein-bound iodine oncentration was raised to levels considerably above normal.

METHODS

The method of Dempsey and Astwood (1) was used to determine the amount of odium-*l*-thyroxine injected subcutaneously daily which prevented goiter in rats fed diets containing propylthiouracil in 0.03% concentration. The thyroxine was dissolved n a slightly alkaline isotonic saline solution. The doses ranged from 1 μg. to 5 μg. per at per day. The temperature of the animal room was 70 to 75° F.

Diet A consisted of gluten flour, 300 gm.; vitamin-free casein, 110 gm.; corn oil, 80 gm.; entire wheat flour, 100 gm.; sucrose, 370 gm.; salt mixture, 50 gm. The salt mixture contained the following: calcium carbonate, 300 gm.; potassium phosphate, dibasic, 150 gm.; magnesium sulfate, 50 gm.; sodium chloride, 500 gm.; ferrous sulfate, 1.0 gm.; copper sulfate, 0.05 gm.; manganese sulfate, 0.05 gm.; cobalt chloride, 0.05 gm.; sodium molybdate, 0.05 gm.; and zinc sulfate, 0.05 gm. A mixture of water-soluble vitamins was added. The iodine content was about 0.05 μg. per gm. of diet.

Diet B contained: corn starch, 270 gm.; sucrose, 270 gm.; casein, 260 gm.; corn oil, 80 gm.; salt mixture, 50 gm.; vitamin mixture, 50 gm.; and sodium iodide was added to make the iodine content 0.2 μg./gm. diet.

The third diet was Purina mink chow,[3] a high residue diet (2).

An experiment was made in which thyroxine was tagged initially with 30 μc. I^{131}-labelled thyroxine per 100 ml. of a solution containing 2.0 mg. of sodium-*l*-thyroxine. The purpose was to determine the plasma concentration of bound iodine by isotopic dilution, the method being similar to that reported for iodine metabolism studies (3). This provided an independent method for determining protein-bound iodine concentrations in plasma, based on the assumption of a state of equilibrium in which the specific activity of the plasma thyroxine was the same as that of the I^{131}-labelled stock solution of sodium-*l*-thyroxine.

Propylthiouracil and thyroxine were given for 10 to 14 days. The rats were anesthetized with ether 24 hours after the last injection of thyroxine. Blood was withdrawn from the inferior vena cava for determination[4] of protein-bound iodine by the method of Zak *et al.* (4) as modified by Astwood *et al.* (5). The thyroid glands were excised and weighed on a torsion balance.

RESULTS

Tables 1 and 2 indicate that the goiter-preventive dose of sodium-*l*-thyroxine was between 2.5 and 5.0 μg. per rat per day. However, with each level of thyroxine dosage the feeding of the low residue Diet B resulted in plasma concentrations of protein-bound iodine 1.3 to 2 times greater than were found with Purina chow. With the exception of two rats taking Diet

[3] Iodine analyses on Purina mink chow showed 0.4 μg./gm. when 0.2 to 0.5 gram was analyzed directly (5).

[4] The technical assistance of Rosemary Libby, Howard Libby and Shirley Miller is gratefully acknowledged.

TABLE 1

Average body weight (No. rats)	Thyroxine dose, μg./day	After two weeks of thyroxine	
		Thyroid, mg./100 gm. body weight	Plasma bound iodine, μg./100 ml.
Diet A plus 0.03% propylthiouracil			
177 (Propylthiouracil not given, 5)	0	6.8±1.23*	5.5±0.35
161 (5)	0	28.2±10.3	2.5±0.95
149 (4)	1.3	20.2±2.2	5.2±0.6
149 (3)	2.5	17.0±2.5	7.5±0.4
148 (4)	5.0	8.4±3.3	11.1±2.2
Diet B plus 0.03% propylthiouracil			
162 (3)	1.3	17.0±3.8	7.1±0.6
160 (3)	2.5	14.6±4.6	9.3±1.2
166 (3)	5.0	5.3±0.8	16.8±2.6
Diet C (Purina) plus 0.03% propylthiouracil			
161.2 (Propylthiouracil not given, 10)	0	5.1±1.0	5.1±1.32
179 (8)	0	22.3±5.5	2.9±0.68
134 (7)	1.3	16.0±3.0	5.4±0.8
143 (6)	2.5	15.0±2.5	6.9±1.6
142 (4)	5.0	6.7±1.3	8.8±2.7

* Standard error of the mean.

B (Table 2) the rats injected with less than 5 μg. thyroxine per day had goiters despite concentrations of protein-bound iodine above the levels found in the untreated rats.

In the experiments recorded in Table 2 the concentration of protein-bound iodine was determined isotopically. It was considered that, after 10

TABLE 2

Body weight in grams (No. rats)	Thyroxine, μg./day	Thyroid, mg./100 gm. body weight	Plasma bound iodine,* μg./100 ml.
Diet B plus 0.03% propylthiouracil for 10 days			
131 (2)	1.2	15.0	5.1
134 (2)	2.5	9.0	10.2
134 (2)	5.0	8.0	16.5
Diet C (Purina) plus 0.03% propylthiouracil for 10 days			
139 (2)	1.2	19.0	2.8
134 (2)	2.5	12.0	4.8
153 (2)	5.0	6.0	7.9

* Plasma thyroxine/100 ml. = (PBI^{131} counts/min./100 ml. plasma) ÷ (counts/min./μg. thyroxine in solution of radiothyroxine at time of radioactive determination)

Bound iodine is 63.6% of calculated sodium thyroxine.

days of administration of propylthiouracil and injections of radio-thyroxine, the plasma concentration of protein-bound iodine would be largely due to injected thyroxine. The specific activity ($I^{131}/\mu g.$ thyroxine) in plasma would approach that of the solution itself. Therefore, the determination of I^{131} in the trichloracetic acid precipitate of plasma would indicate a minimum value for the concentration of protein-bound iodine. The table indicates that rats which received 1.2 $\mu g.$ to 2.5 $\mu g.$ sodium-*l*-thyroxine per day developed goiters from propylthiouracil when the plasma concentration of protein-bound iodine was normal. In Table 2 the only rats which had lowered plasma concentrations of protein-bound iodine were those fed Purina chow and injected with 1.2 $\mu g.$ *l*-thyroxine per day. Feeding the low residue diet to rats given daily injections of thyroxine appears to have resulted in plasma concentrations of protein-bound iodine twice those found when the high residue Purina chow diet was fed.

DISCUSSION

This study shows that goiter prevention in propylthiouracil-treated rats is only achieved through daily injections of sodium-*l*-thyroxine in amounts which elevate the plasma concentration of protein-bound iodine to values 2 to 3 times normal. This clearly raises the possibility that the goiter-prevention method of Dempsey and Astwood (1) gives an erroneously high value for the daily secretion rate of thyroxine by the thyroid gland. Taurog and Chaikoff (6), using a method involving specific activities of diiodotyrosine and thyroxine in the thyroid gland, calculated a thyroxine secretion rate of 1.5 $\mu g./100$ gm. body weight per day, in contrast to the 5.2 $\mu g.$ figure of Dempsey and Astwood. These two groups used different diets.

If, under the conditions of these experiments, protein-bound iodine determinations provide a measure of circulating thyroid hormone, it is likely that goiter has developed under conditions in which thyrotropin production is normal. Goldberg *et al.* (7) have reviewed briefly the point of view that thyroid-pituitary relationships are not fully explained by a simple feedback mechanism, and the present study provides further support for this contention. It was first suggested by Albert *et al.* (8) and confirmed by Halmi and Spirtos (9) that the effects of thyrotropin on thyroid cell height were enhanced by antithyroid drugs. Rawson (10) has taken the position that antithyroid drugs cause goiter through two mechanisms: by enhancing thyrotropin output in response to hypothyroidism and by potentiating the effect of thyrotropin. This formulation allows the inference that goiter may occur despite some suppression in thyrotropin production, and this study provides a basis for exploring this possibility. Other manipulations which deplete thyroid iodine stores have in common with propylthiouracil the augmentation of iodide concentrating activity (11, 12). It seems likely that goitrogenesis due to feeding of propylthiou-

racil to intact rats having high protein-bound iodine concentrations, as observed in this study, is due directly to an increased thyroid sensitivity to thyrotropin.

Among possible objections to attaching significance to these findings is the non-specificity of protein-bound iodine in plasma as a measure of circulating thyroid hormone. With the low iodine diets this objection seems less significant than in the experiments in which dietary iodine was higher. Objection also may be held on the score that normally triiodothyronine is also a component of the protein-bound iodine. However, Gross (13) has found that the concentration of triiodothyronine in plasma is probably very much less than that of thyroxine and, in addition, Gross and Leblond (14) found Compound No. 1, subsequently shown to be triiodothyronine (15), in the plasma of thyroidectomized rats injected with thyroxine.

REFERENCES

1. Dempsey, E. W. and E. B. Astwood: *Endocrinology* **32**: 509. 1943.
2. Van Middlesworth, L.: *Endocrinology* **61**: 570. 1957.
3. Van Middlesworth, L.: *Endocrinology* **58**: 235, 1956.
4. Zak, G., H. H. Willard, G. B. Myers and A. J. Boyle: *Anal. Chem.* **24**: 1345. 1952.
5. Astwood, E. B., C. E. Cassidy, M. S. Raben and S. M. Astwood: Ciba Foundation Colloquia on Endocrinology, Vol. 11, 95, 1957.
6. Taurog, A. and I. L. Chaikoff: *J. Biol. Chem.* **169**: 49, 1947.
7. Goldberg, R. C., J. Wolf and R. O. Greep: *Endocrinology* **60**: 38, 1957.
8. Albert, A., R. W. Rawson, C. Riddell, P. Merrill and B. Lennon: *Endocrinology* **40**: 361. 1947.
9. Halmi, N. S. and B. N. Spirtos: *Endocrinology* **55**: 613. 1954.
10. Rawson, R. W.: Ciba Foundation Colloquia on Endocrinology, Vol. 4, 294. 1952.
11. Halmi, N. S., B. N. Spirtos, E. M. Bogdanove and H. J. Lipner: *Endocrinology* **52**: 19, 1953.
12. VanderLaan, W. P. and R. Caplan: *Endocrinology* **54**: 437. 1954.
13. Gross, J.: Brookhaven Symposia in Biology, No. 7, *The Thyroid*, p. 102, 1954.
14. Gross, J. and C. P. Leblond: *Proc. Soc. Exper. Biol. and Med.* **76**: 686, 1951.
15. Gross, J. and R. Pitt-Rivers: *Lancet* **1**: 439. 1952.

STUDIES IN THE ACTIVATION AND INHIBITION OF ADRENOCORTICOTROPHIN SECRETION

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ABSTRACT

Comparisons have been made in the depletion of adrenal ascorbic acid which resulted from cold, hypertonic saline and bell-ringing, in normal, neurohypophysectomized and reserpine injected male rats. The effects upon ACTH/AAF release of the stimulation of neurohypophysial hormone release, or its injection, were recorded in normal animals and neurohypophysectomized animals subjected to cold stress. Also, the effects of neurohypophysectomy and reserpine injection upon the adrenal weights of normal and adrenal-enucleate-regenerate animals were recorded.

It was concluded that:

1. The liberation of AAF by the adenohypophysis seems to necessitate the concomitant release of neurohypophysial hormone or something associated with it and it is suggested that the release of ADH by the neurohypophysis results in an "obligatory" release of AAF.
2. The neurohypophysis is involved in the release of ACTH/AAF in response to systemic stresses but not to emotional ones. It is suggested that separate centres within the hypothalamus control, via different pathways, the secretion of AAF by the adenohypophysis.
3. The hypertrophy of the adrenals which results from neurohypophysectomy, and the regeneration of the adrenals after enucleation, are discussed and related to the results of other workers. It is suggested that enucleated adrenals regenerate under the influence of a basal secretion of ACTH/AWF, the control of which probably does not involve hypothalamic centres and that the release of a hypertrophy AWF involves a hypothalamic centre the stimulation of which depends upon the capacity of the centre/s which stimulate/s AAF release, to meet body requirements.

THE mechanisms involved in the release of ACTH from the pituitary are, to say the least, complex. ACTH itself appears to have a dual function in that it maintains adrenal weight and stimulates an increase in the release of steroids from the adrenal cortex (1) and these two functions are obviously related to one another since adrenocortical volume varies according to the demands made upon its productive capacity. In addition, it has become customary to refer to two categories of stress, both of which result in an increased secretion of ACTH (2). These are, neurotropic or emotional stress which activates the anterior lobe of the pituitary via the central nervous system and hypothalamus, and metabolic or systemic

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stress which generally results in a more direct action of blood-bourne agents upon the anterior lobe but which may also involve the central nervous system (3, 4, 5 and 6). Since de Groot and Harris (7) postulated a chemical transmitter passing from the hypothalamic area via the hypophysial portal vessels to stimulate the anterior lobe, many workers have attempted to relate this unknown agent to the Gomori-positive neurosecretory material demonstrated in the hypothalamic nuclei and known to be intimately related to the functioning of the neurohypophysis(8).

The present experiments were designed in order to attempt to relate the function of the neurohypophysis to the release of ACTH by the adenohypophysis. They involve the stimulation or inhibition of one or both of these organs with the above mentioned findings in mind, and the extent to which the results shed light upon the control of ACTH secretion is discussed.

MATERIAL AND METHODS

Male albino rats of the Wistar strain were used in all experiments. They were kept at a temperature of about 70° F and fed on commercial rat cake and water *ad lib.* The percentage depletion of adrenal ascorbic acid resulting from any stress and the percentage adrenal weight at the end of the experimental period were used as criteria for comparing the amount of ACTH (Ascorbic acid factor, AAF, and Adrenal weight factor, AWF, respectively) liberated by the pituitary under the various experimental conditions. The experimental groups were divided up as follows:

Blockage of ACTH (AAF) secretion

Normal control animals subjected to stress

a. Cold. Those rats to be subjected to stress were placed in the cold room for 1 hour at 30° F and sacrified 1 hour after the stress.
b. Bell ringing. Animals to be stressed were placed in a separate room and subjected to the ringing of a loud electric bell for a period of 10 minutes. They were sacrificed half an hour after the stress.
c. Hypertonic saline. The animals to be stressed received an infusion of 5 ml. of warm 308 mM saline over a period of 3 minutes and were sacrified an hour later. Earlier experiments designed to induce the secretion of ACTH by the administration of hypertonic saline solutions via stomach tube, proved to be abortive, since the act of inserting the stomach tube provided an emotional stress which was sufficient to mask the effect of the saline. Thus in this and all other experiments involving saline infusion, a polythene canula was inserted into the jugular of both unstressed and stressed animals 48 hours before the infusion and the unstressed animals were killed 1 hour after a mock infusion.

Neurohypophysectomized animals subjected to stress

The parapharyngeal approach was used in the removal of the posterior lobe of the pituitary and the anterior lobe was only very slightly damaged. Normal increase in body weight and adrenal hypertrophy, which follow this operation, confirm that the anterior lobe continues to function. All stress experiments were performed 10 days after the operation, during which period the rats drank 4-5 times normal quantities of water. The depletion of adrenal ascorbic acid, which resulted from the subjection of these rats to the stresses of cold, bell-ringing and hypertonic saline, was compared with the corresponding figure in the normal control animals.

Animals injected with reserpine and subjected to stress

All rats due to be stressed received a subcutaneous injection of 1 mg./kg. body weight of resperpine the day before the experiment and the same dose was given half an hour before stressing. The experiments were performed simultaneously with those on the normal animals and the same three types of stress were imposed. Using the same unstressed controls the depletion of adrenal ascorbic acid was again compared with that obtained in the normal animals.

Stimulation of ACTH (AAF) secretion

In considering the relationship between posterior and anterior lobe secretion, the following experiments were designed in order to stimulate or to substitute for posterior lobe secretions and to relate the resulting effects to ACTH secretion:

Normal animals

a. Intravenous infusion of Tyrode's Ringer solution. 5 ml. of warm Ringer solution were infused, over a period of 3 minutes, into rats which had been canulated 48 hours before and the rats killed 1 hour later.
b. Intravenous infusion of hypertonic saline. The stressed rats received 5 ml. of 308 mM saline (as in c above).
c. Intravenous infusion of Ringer plus Pitressin. 0.3 U. of Pitressin (Parke, Davis) was added to the Ringer solution and the whole made up to 5 ml.
d. Intravenous infusion of Ringer plus Pitocin. 0.3 U. of Pitocin (Parke, Davis) was added to the Ringer solution and the whole made up to 5 ml.
e. Intravenous infusion of Ringer plus nicotine. Two mg./kg. body weight of nicotine were added to the Ringer solution and the whole made up to 5 ml.

Neurohypophysectomized animals subjected to cold stress

The procedure followed that for neurohypophysectomized animals subjected to cold stress (see above) except that the stressed animals received a subcutaneous injection of one of the following substances half an hour before they were placed in the cold room.

a. 0.3 U. of Pitressin
b. 0.3 U. of Pitocin
c. 2mg./kg. body weight of nicotine.

Animals injected with reserpine and subjected to the stress of bell-ringing

The procedure followed that for resperpine injected animals subjected to bell-ringing (see above) except that a second group of stressed animals received 0.3 U. of Pitressin together with 1 mg./kg. of resperine half an hour before stressing.

Hypophysectomized control animals

These animals were sacrificed 1 hour after they received a subcutaneous injection of 0.3 U. of Pitressin plus 0.3 U. of Pitocin.

The influence of neurohypophysectomy and reserpine upon ACTH/AWF secretion

In the course of this and previous work it was found that neurohypophysectomy resulted in a marked increase in adrenal weight, presumably due to an increased secretion of ACTH/AWF. It is also generally accepted that the secretion of this hormone is increased after adrenal enucleation (9) as it is after adrenalectomy (10) and prolonged stress (11). The adrenal weights of the animals subjected to the following conditions were recorded in an attempt to relate the influences of neurohypophysectomy and reserpine to adrenal weight.

a. Neurohypophysectomized normal animals. Adrenal weights were taken 10 days after the operation.

b. Neurohypophysectomized adrenal-enucleate-regenerates. Neurohypophysectomy was performed 1 day before adrenal enucleation. Both adrenals were enucleated in this and all other enucleate groups mentioned and all adrenals were allowed to regenerate for 7 days.

c. Reserpine injection of adrenal-enucleate-regenerates. Reserpine (1 mg./kg.) was injected daily throughout the 7 day period of regeneration.

d. Reserpine injection of adrenal-enucleate-regenerates stressed by bell ringing. An electric bell was placed immediately above the cages and set to ring for 55 seconds every hour throughout the 7 days of regeneration. Reserpine (1 mg./kg.) was injected daily throughout this 7 day period.

e. Reserpine injection of neurohypophysectomized animals. Reserpine (1 mg./kg.) was injected daily throughout the 10 day experimental period.

RESULTS

Blockage of ACTH (AAF) secretion

The effect of the removal of the posterior lobe of the pituitary upon the response of the anterior lobe to the stresses of cold, bell ringing and hypertonic saline are shown in Table 1. The figures for the percentage depletion of adrenal ascorbic acid indicate that this operation reduces the release of ACTH/AAF by the adenohypophysis in response to the stresses of cold and the intravenous infusion of hypertonic saline but not to the stress of bell ringing. The reverse is true after reserpine injection since the response to both cold stress and hypertonic saline infusion remained normal whilst the response to bell ringing was abolished altogether.

Stimulation of ACTH (AAF) secretion

The effects of the attempted stimulation of ACTH secretion in normal, neurohypophysectomized and hypophysectomized animals are shown in Table 2. In the normal animals the infusion of 5 ml. of Tyrode's Ringer solution resulted in a 7.6% depletion of adrenal ascorbic acid ($P = >0.3 < 0.4$). However, when 308 mM saline was substituted for

TABLE 1. THE EFFECT OF COLD (1 HOUR AT 30° F), BELL RINGING (10 MINUTES) AND INTRAVENOUS 308 MM SALINE UPON THE PERCENTAGE ASCORBIC ACID CONTENT OF THE ADRENALS IN NORMAL, NEUROHYPOPHYSECTOMIZED AND RESERPINE INJECTED (1 MG./KG. BODY WT.) RATS

Category of rat	Treatment of stressed animals	Ascorbic acid mg./100 gm. of adrenal tissue ± S.E.		% depletion on stress	'P' value
		Unstressed	Stressed		
Normal	Cold	423.7 ± 23.7 (8)	317.2 ± 13.7 (9)	25.1	<0.01
	Bell ringing	408.5 ± 13.1 (6)	338.5 ± 13.2 (6)	17.1	<0.01
	308 mM saline	316.1 ± 16.8 (10)	235.4 ± 10.6 (9)	25.5	<0.01
Neurohypophysectomized	Cold	369.8 ± 20.1 (9)	365.6 ± 18.2 (9)	1.1	>0.8
	Bell ringing	398.0 ± 17.7 (7)	323.1 ± 18.7 (8)	18.8	0.01
	308 mM saline	322.8 ± 19.0 (7)	306.1 ± 13.0 (7)	5.1	<0.5>0.4
Normal	Injected with 1 mg./kg. reserpine and subjected to				
	Cold	423.7 ± 23.7 (8)	317.0 ± 14.5 (8)	25.1	<0.01
	Bell ringing	408.5 ± 13.1 (6)	426.6 ± 13.0 (6)	4.4 incr.	—
	308 mM Saline	316.1 ± 16.8 (10)	247.4 ± 14.5 (7)	21.7	<0.01

( ) No. of animals.

TABLE 2. THE EFFECT OF NEUROHYPOPHYSIAL STIMULATION AND THE ADMINISTRATION OF NEUROHYPOPHYSIAL HORMONES (PARKE, DAVIS PITRESSIN AND PITOCIN) UPON THE ASCORBIC ACID CONTENT OF THE ADRENALS OF VARIOUS CATEGORIES OF RATS

Category of rat	Treatment of stressed animals	Ascorbic acid—mg./100 gm. of adrenal tissue ± S.E.		% depletion on stress	"P" value
		Unstressed	Stressed		
Normal	*Infusion of:*				
	Ringer	305.7 ± 15.2 (8)	282.5 ± 15.4 (8)	7.6	>0.3<0.4
	308 mM saline	316.0 ± 11.6 (12)	251.0 ± 13.2 (10)	20.6	<0.01
	Ringer +0.3 U. Pitressin	347.2 ± 14.7 (9)	263.7 ± 19.7 (7)	24.0	<0.01
	Ringer +0.3 U Pitocin		260.7 ± 20.7 (7)	25.0	<0.01
	Ringer +2 mg./kg. Nicotine	299.4 ± 15.2 (9)	223.8 ± 6.7 (8)	25.2	<0.01
Neurohypophysectomized	*Cold Stress and injection of:*				
	0.3 U. Pitressin	496.0 ± 26.4 (10)	375.6 ± 6.1 (9)	24.2	<0.01
	0.3 U. Pitocin	516.0 ± 28.0 (9)	412.2 ± 27.0 (9)	20.1	<0.02>0.01
	2 mg./kg. Nicotine	495.7 ± 26.0 (7)	401.1 ± 22.0 (7)	19.0	<0.02>0.01
Normal	*Stressed by bell ringing and injected with:*				
	1 mg./kg. Reserpine	381.1 ± 22.2 (6)	376.0 ± 13.1 (7)	1.3	>0.8
	1 mg./kg. Reserpine plus 0.3 U. Pitressin		277.0 ± 13.9 (7)	27.3	<0.01
Hypophysectomized	*Injection of:*				
	0.3 U. Pitressin plus 0.3 U. Pitocin	367.5 ± 28.7 (8)	375.4 ± 17.1 (8)	—	

( ) No. of animals.

Ringer solution or when Pitressin, Pitocin or nicotine were added to the Ringer solution, the depletion of adrenal ascorbic acid became significant—around the level of 24%.

The adrenal ascorbic acid content in the unstressed animals of groups which had been subjected to jugular canulation in preparation for infusion was lower than in non-canulated groups. This is probably due to the stressing effects of canulation and reflects an increased output of ACTH in these groups. The significance of this control level of adrenal ascorbic acid has been discussed previously (9).

In the neurohypophysectomized animals, where cold stress had resulted in the insignificant depletion of adrenal ascorbic acid of 1.1% (P = >0.8) the injection of Pitressin, Pitocin or nicotine, before the stress was applied, resulted in a significant depletion (around 21%) in each case.

Also, in normal animals which showed a negligible depletion of adrenal ascorbic acid in response to bell ringing, when pre-treated with reserpine, the addition of 0.3 U. of Pitressin to the injected reserpine resulted in a normal depletion of 27.3%.

Hypophysectomized animals acted as controls for the above groups and in these animals the injection of a solution containing both Pitressin and Pitocin produced no depletion of adrenal ascorbic acid.

The influence of Neurohypophysectomy and reserpine upon ACTH(AWF) secretion

The final percentage adrenal weights of the various categories of rats used in these experiments are shown in Table 3. After 10 days, during which time they drank 4 to 5 times normal quantities of water, neurohypo-

physectomized animals showed a percentage adrenal weight of 25.23 mg. as against 15.61 mg. in the normal animal. The daily injection of 1 mg./kg. of reserpine did not prevent this increase in adrenal size. Bilaterally enucleated adrenals in the normal animal regenerated to a weight of 11.2 mg.% in 7 days and similar animals which had been previously neurohypophysectomized, although drinking similar quantities of liquid (Figure 1), showed a regeneration weight of 21.9 mg. %. Lastly, the regenerated adrenals of animals which were subjected to bell ringing

TABLE 3. THE INFLUENCE OF NEUROHYPOPHYSECTOMY AND RESERPINE UPON ADRENAL WEIGHT

Treatment	No. of rats	Final average body weight, gm. ±S.E.	Adrenal weight—mg./100 gm. body ±S.E.	
			Controls	Neurohypophysectomized
Normal	39	224.6±2.9	15.61±0.78	—
	19	210.5±2.5	—	25.23±1.02
Adrenal-enucleate-regenerate	9	173.6±3.6	11.2 ±0.736	—
	6	133.3±3.5	—	21.9 ±2.1
			Controls	Reserpine injected, 1 mg./kg. daily
Adrenal-enucleate-regenerate	17	141.3±2.1	10.38±0.47	—
	10	120.6±4.7	—	12.03±1.12
Adrenal-enucleate-regenerate stressed by bell ringing	7	142.1±4.5	17.35±1.24	—
	7	133.1±4.5	—	13.0 ±0.73
Neurohypophysectomized	6	153.0±8.4	27.9 ±3.28	—
	9	175.5±7.6	—	33.9 ±2.6

throughout the period of regeneration were heavier (17.35 mg. %) than those of the control animals (10.38 mg. %) and the daily injection of 1 mg./kg. of reserpine, which had no significant effect upon the regeneration of the control adrenals (12.03 mg. %), did prevent the hypertrophy which had resulted from bell ringing (13.0 mg. %).

DISCUSSION

The present findings on the relationship between hormones of the neurohypophysis and ACTH secretion are in agreement with those of Sobel, Levy, Marmorston and Rosenfeld (12), Martini and DePoli (13) and others. The administration of Pitressin or Pitocin induced a significant depletion of adrenal ascorbic acid in animals which had not otherwise shown such a response. Also, the induction of ADH release, by the administration of nicotine (see 14) and hypertonic saline (see 15), produced similar depletions of ascorbic acid in stressed animals which had shown only slight responses in the absence of these stimulating substances. That this ACTH

release is not due to any necessity for additional corticosteroids at the kidney level was demonstrated, firstly by the finding that little ACTH was released when hypertonic saline was administered to neurohypophysectomized animals with an otherwise functional anterior lobe and secondly, by the finding that ACTH was not released when a Ringer load alone was administered, but that the addition of Pitressin, Pitocin or nicotine to the Ringer did cause a depletion of adrenal ascorbic acid. This latter reaction must be mediated via the pituitary since no response to Pitressin and Pitocin injection was obtained in hypophysectomized animals.

Thus the presence of posterior lobe hormones (or something associated

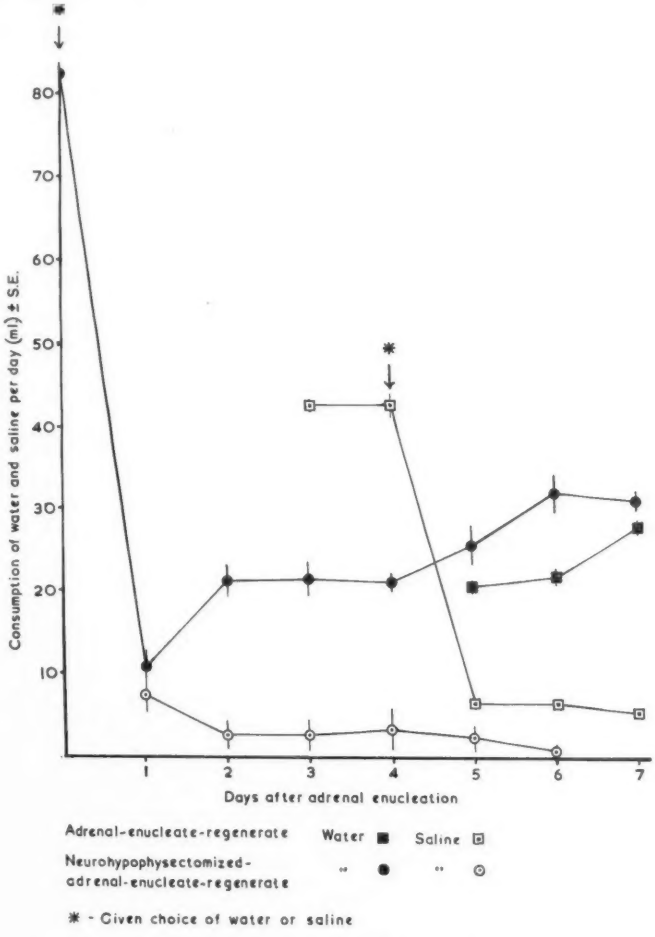

FIG. 1. Consumption of water and saline (0.9%) by adrenal-enucleate-regenerate and neurohypophysectomized-adrenal-enucleate-regenerate rats.

with them) in the circulation appears to be essential to ACTH release, and, moreover, it is suggested that the presence of these hormones at the level of the pituitary results in a direct "obligatory" release of ACTH. On the other hand, it would appear that mechanisms which are known to result in the release of ACTH also involve the concomitant release of ADH, as in emotional stress (16 & 17) or systemic stress (15). However, the above experiments suggest the existence of two distinct mechanisms of ACTH release. One of these mechanisms, which responds to emotional stress, may, at some unknown level, be stimulated by nicotine and can be blocked by reserpine. The other responds to systemic stresses such as cold and hypertonic saline and, unlike the former, necessitates the presence of an intact neurohypophysis. These findings agree generally with results of corresponding work on ADH release reported by Dingman (18), Dingman and Thorn (19) and Dingman and Despointes (20) who concluded that nicotine stimulated hypothalamic centres to release ADH in patients with diabetes insipidus, whereas hypertonic saline did not. They also found that, in normal subjects, the nicotine induced decrease in free water clearance was blocked by cortisone, hydrocortisone and prednisolone, whereas that to hypertonic saline and Pitressin was not.

Thus, although these two mechanisms of stress response have something in common—the release of posterior lobe hormone—the present experiments suggest the following conclusion. That emotional stresses involve the passage of neurosecretory material, possibly from nuclei in the anterior region of the hypothalamus, to the adenohypophysis, via the main portal vessels, so that neurohypophysectomy would not interfere with this mechanism. That systemic stresses involve the passage of such material, possibly from more posterior nuclei of the hypothalamus, to the adenohypophysis, via other portal vessels within posterior lobe tissue, so that this pathway would be servered by neurohypophysectomy. These suggestions are strongly supported by the work of Daniel and Prichard (21) who found that in the rat cautery of the hypophysial portal vessels resulted in necrosis of only the central part of the adenohypophysis and that the tissue which did not become necrosed was supplied by uncauterised portal vessels which drain part of the neural lobe.

The relationship between ACTH/AAF and ACTH/AWF secretions, if these can be considered separately, are obviously complex. It is known that, firstly, the weight of an animal's adrenals is, under normal circumstances, related to the weight (22), the surface area (23) and the sex (24 & 25) of the animal; secondly, that the removal of one adrenal results in the compensatory hypertrophy of the other (26 & 27); thirdly, that the demands for corticoids which result from stress bring about an increased secretion of ACTH/AAF and, depending upon the extent of this demand, adrenal hypertrophy (11). These and other findings (9), which attempt to

relate the sex of the animal to the adrenal weight and its capacity to respond to stress, would suggest the existence of a balance to control the ratio of AAF/AWF liberated by the pituitary and which might be regulated by the degree of stimulation or inhibiton of separate but closely associated centres in the hypothalamus.

In the present experiments neurohypophysectomized normal animals, which did not release AAF in response to systemic stress, showed normal body weight increase and a considerable degree of adrenal hypertrophy even when reserpine, which had been shown to block the hypertrophy due to bell ringing, was injected daily. Also, the regeneration weight of enucleated adrenals, during a seven day period, was doubled when animals were previously neurohypophysectomized and this increase occurred in spite of a normal consumption of water. These findings agree with those of McCann and Brobeck (28) who showed that, following lesions of the supra-optico-hypophysial tract, adrenal hypertrophy resulted in cases with high diabetes insipidus. However, this hypertrophy resulted in some cases but not in others. Similarly, Greer and Erwin (29) found that adrenal atrophy resulted from destruction of the medium eminence in some cases, but in others there was no correlation between destruction of the supra-optic and paraventricular nuclei and the absence of amphenone-induced adrenal hypertrophy. They suggested, therefore, that Pitressin may not play a significant role in the control of corticotrophin secretion. If, as previously suggested, separates centres *are* responsible for the release of AAF and AWF, then the release of AAF might depend upon the degree of hypothalamic stimulation balanced against the titers of blood corticoids, and the release of AWF upon the degree of hypothalamic stimulation plus the capacity of AAF secretion to meet this balance. Adrenal atrophy, which follows corticosteroid administration, would, on this hypothesis, be due to the fact that AAF and hence AWF, were no longer required. Also, the presence of high blood corticoids together with adrenal hypertrophy, as is found in continuous stress, could be accounted for.

On this hypothesis too, the findings of McCann and Brobeck and Greer and Erwin could be explained. A median eminence lesion might destroy either the centre responsible for the stimulation of AAF release or that for AWF release or, if they are closely related, both, so that ambiguous results may be obtained. In connection with Greer and Erwin's suggestion that Pitressin may not play a significant role in the control of corticotrophin secretion, the present data would suggest that this hormone is associated with the release of AAF only, so that if the supra-optic and paraventricular nuclei are responsible for the release of AAF, then the destruction of these nuclei might *induce* adrenal hypertrophy. A similar reaction is suggested for the adrenal hypertrophy in neurohypophysectomized rats in which the release of AAF in response to systemic demands was blocked. This hyper-

trophy tended to be even greater when, in addition, the response to emotional stresses was blocked by reserpine.

In addition to the consideration of an ACTH factor responsible for adrenal hypertrophy the findings in adrenal-enucleate animals are of interest since they agree with those of Ganong and Hume (30) who suggest that the pituitary maintains a basal secretion of ACTH in the presence of lesions of the median eminence which abolish compensatory and stress-induced adrenal hypertrophy. It appears that the adrenals regenerate, under the influence of this basal secretion, to the percentage weight of 10–11 mg. This secretion is not blocked by reserpine, but just as Ganong and Hume prevented stress-induced adrenal hypertrophy by hypothalamic lesions, so I blocked this hypertrophy by the injection of reserpine. Schneider, Holden, Rinehart and Moore (31) showed that reserpine probably blocks afferent impulses and does not depress centres within the hypothalamus, so that although the mechanism whereby this hypertrophy was blocked probably differed in the two cases, a distinction between the mechanisms involved in the release of the basal secretion on the one hand and the factor responsible for adrenal hypertrophy on the other, is indicated.

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REFERENCES

1. Dixon, H. B. F., M. P. Stack-Dunne, F. G. Young and D. B. Carter: *Nature* London **168:** 1084. 1951.
2. Fortier, C.: *Endocrinology* **49:** 782. 1951.
3. Hume, D. M.: Ciba Foundation Colloquia on Endocrinology **4:** 87. 1952.
4. Porter, R. W. *Am. J. Physiol.* **169:** 629. 1952.
5. Porter, R. W.: *Am. J. Physiol.* **172:** 515. 1953.
6. Porter, R. W.: *Recent Progr. Hormone Res.* **10:** 1. 1954.
7. Grogt, J. de and G. W. Harris: *J. Physiol.* **111:** 335. 1950.
8. Scharrer, E. and B. Scharrer: *Recent Progr. Hormone Res.* **10:** 183. 1954.
9. Nowell, N. W. and I. Chester Jones: *Acta Endocrinol.* **26:** 273. 1957.
10. Gemzell, C. A., D. C. van Dyke, C. A. Tobias and H. M. Evans: *Endocrinology* **49:** 325. 1951.
11. Sayers, G. and M. A. Sayers: *Ann. New York Acad. Sc.* **50:** 596. 1949.
12. Sobel, H., R. S. Levy, J. Marmorston, S. Schapiro and S. Rosenfeld: *Proc. Soc. Exper. Biol. & Med.* **89:** 10. 1955.
13. Martini, L. and A. DePoli: *J. Endocrinol.* **13:** 229. 1956.
14. Burn, J. H., L. H. Truelove and I. Burn: *Brit. M. J.* **1:** 403. 1945.
15. Verney, E. B.: *Proc. Roy. Soc.* London B. **135:** 25. 1947.
16. O'Connor, W. J. and E. B. Verney: *Quart. J. Exper. Physiol.* **31:** 393. 1942.

17. O'Connor, W. J.: *Quart. J. Exper. Physiol.* **33:** 149. 1946.
18. Dingman, J. F.: *Fed. Proc.* **13:** 36. 1954.
19. Dingman, J. F. and G. W. Thorn: *J. Clin. Endocrinol. and Metab.* **15:** 871. 1955.
20. Dingman, J. F. and R. H. Despointes: *J. Clin. Endocrinol. and Metab.* **16:** 936. 1956.
21. Daniel, P. M. and M. M. L. Prichard: *Quart. J. Exper. Physiol.* **41:** 215. 1956.
22. Donaldson, H. H.: *The Rat* Mem. Wistar Inst. Anat. no. 6, 2nd ed. Philadelphia, 1924.
23. Houssay, B. A. and E. A. Molinelli: *C. R. Soc. Biol.* (Paris) **95:** 819. 1926.
24. Donaldson, J. C.: *Proc. Soc. Exper. Biol. & Med.* **25:** 300. 1928.
25. Chester Jones, I.: *Brit. M. Bull.* **11:** 156. 1955.
26. Sayers, G. and M. A. Sayers: *Recent Progr. Hormone Res.* **2:** 81. 1948.
27. Tepperman, J., F. L. Engel and C. N. H. Long: *Endocrinology* **32:** 373. 1943.
28. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exper. Biol. & Med.* **87:** 318. 1954.
29. Greer, M. A. and H. L. Erwin: *Endocrinology* **58:** 665. 1956.
30. Ganong, W. F. and D. M. Hume: *Endocrinology* **55:** 474. 1954.
31. Schneider, A., E. Holden, R. Rinehart and R. Moore: *Am. J. Physiol.* **181:** 64. 1955.

THE EFFECTS OF ATRIAL STRETCH ON ALDOSTERONE SECRETION[1]

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ABSTRACT

A study was carried out to determine whether the secretion rates of aldosterone and of hydrocortisone are subject to regulation by receptors located in the atria of the heart. In dogs, the right or left atrium was stretched with sutures which extended from the atrium to the thoracic wall. Adrenal venous blood was collected and analyzed for aldosterone and hydrocortisone. Following right atrial stretch the aldosterone secretion rate was significantly reduced. However, stretching the left atrium was without effect on the secretory rate of aldosterone. These findings strongly suggest that the secretion of aldosterone may be regulated in part by stretch receptors located in the right atrium.

SOME years ago J. P. Peters postulated that the secretion of electrolyte-active steroids is under the control of the volume of the vascular system (1). The theory has been widely discussed (2, 3). The demonstration that aldosterone is the principle electrolyte-active steroid of the adrenal cortex stimulated reinvestigation of this concept. Urinary aldosterone levels fall after water-loading and rise after loss of body water (4, 5, 6). Loss of blood has been shown to stimulate aldosterone secretion in the dog (7, 8). The well-documented increase in aldosterone secretion following sodium deprivation (9) has been thought by some workers to be mediated by contraction of body fluid compartments rather than by direct action of the sodium ion (6).

However, the physiological system whereby changes in body fluid volume mediate changes in steroid output has remained unknown. Receptors, afferent pathways and central integrative mechanisms have never been elucidated. Henry and his collaborators have demonstrated that distension of the left atrium in dogs induces a prompt diuresis and have proposed that atrial stretch receptors play a part in the homeostatic regulation of water balance (10). It was of considerable interest to determine whether a similar volume receptor mechanism might exist for the control of electrolyte homeostasis, mediated by the adrenal cortex. Unfortunately the reports of

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these workers do not shed light on the problem. In Henry's experiments the atria were distended for only a few minutes at a time. Significant changes in the rate of steroid secretion occur rather slowly (11). Following complete hypophysectomy the steroid secretion rate remains high for 15 minutes or longer, and then gradually declines, reaching the low rate characteristic of hypophysectomy only after approximately one hour. Steroid mediated changes in urinary electrolytes would not be anticipated in the short time periods studied by Henry and his coworkers. Further, the technique of placing balloons in the atria (which introduces the complication of reducing cardiac output) does not permit study of the effects of longer-term distension of the atrium. Accordingly, a simple technic was devised to stretch the atria without the introduction of balloons. The results indicate that the right atrium contains receptors which when subjected to stretch act to inhibit the secretion of aldosterone. It is postulated that this system may function to control steroid secretion in response to changes in the volume of blood in the right atrium.

METHODS

Eighteen male mongrel dogs weighting 15.5 to 26.5 kg. were divided among four experimental groups as follows: controls (4), open chest shams (4), open chest plus left atrial stretch (5), and open chest plus right atrial stretch (5). In each experiment the animal was anesthetized with sodium pentobarbital and an external jugular vein and a common carotid artery cannulated for subsequent infusions and mean arterial blood pressure determinations. In the four control experiments the left lumbo-adrenal vein was cannulated and adrenal venous blood was collected for a two hour period. In the other three groups of animals the chest was opened by a transverse incision in the left third or fourth intercostal space and alternating positive pressure artificial respiration was instituted. An incision was made in the pericardium ventral to the left phrenic nerve of sufficient length to expose either the right or left atrial appendage. The cut edges of the pericardium were elevated and secured to the chest wall.

For right atrial stretch, a suture was passed through the tip of the atrial appendage. Similar sutures were placed between the base of the appendage and the sulcus terminalis, one near the entrance of the superior vena cava and the second near the entrance of the inferior vena cava. Sufficient tension was applied to the sutures to visibly stretch the atrial wall in a circumferential manner, without changing the general orientation of the heart structures. The free ends of the sutures were then secured to the wound edges to maintain the tension.

For left atrial stretch, three sutures were also employed, one at the tip of the atrial appendage and the second and third near the openings of the pulmonary veins on opposite sides of the base of the appendage. In the sham-operated animals, sutures were placed without tension in the right atrium (2 dogs) and in the left (2 dogs). In each experiment the exposed portion of the heart was covered with gauze moistened with warmed saline.

One hour following stretch of the right or left atrium, or placing of sutures in the sham-operated groups, the left lumbo-adrenal vein was cannulated and adrenal venous blood collected for a two-hour period. Periodic rectal temperature and mean arterial blood pressure observations were made. The temperature was maintained between 35 and 40° C with heat lamps. Arterial blood pressure was supported when it fell below 80 mm. Hg by infusing 0.2% norepinephrine in 5% glucose. Heparin (100 mg.) was in-

jected intravenously just prior to the cannulation of the adrenal vein to prevent clotting.

The adrenal venous effluent was collected in vessels at 0° C. Immediately following the collection period the sample was diluted with an equal volume of water and extracted with a volume of chloroform equal to that of the blood-water mixture or frozen for subsequent extraction. Aldosterone and hydrocortisone were isolated by paper chromatographic methods (12) and quantitated by absorption at 240 mμ. in methanol. Evidence for the identity of aldosterone, which was isolated as the diacetate, was obtained by mixed chromatograms with authentic aldosterone diacetate, and by bioassay following hydrolysis (acetylcholinesterase) (12). The isolated material was indistinguishable from authentic aldosterone by these criteria.

RESULTS AND DISCUSSION

The results of these studies are summarized in Table 1.

Random selection of animals resulted in an equal distribution of body weights among the various experimental groups.

The mean arterial blood pressure tended in general to fall during the course of the experiments, requiring norepinephrine infusion in some instances. Three animals of the left atrial stretch group, 3 animals in the right atrial stretch group, 1 animal in the sham-operated group, and 2 of the intact animals received norepinephrine during the course of the experiment (Table 1). Examination of the steroid secretion rates in those animals receiving the sympathetic amine in the various groups failed to reveal any consistent evidence of a stimulating action of the drug on steroid secretion. This is in agreement with the findings of Newman and co-workers (13) that

TABLE 1. BODY WEIGHT, ARTERIAL BLOOD PRESSURE, VOLUME OF ADRENAL VENOUS BLOOD, BODY TEMPERATURE, AND STEROID SECRETION IN CONTROL, SHAM, AND ATRIAL STRETCH DOGS

Experimental group	Animal number	Body wt., kg.	Average arterial b.p. during collection period, mm. Hg.	Volume of Adrenal venous blood, ml.	Average body temperature during collection period, ° C	Hydrocortisone secretion, μg./kg. body wt./hr.	Aldosterone secretion, μg./100 kg. body wt./hr.
Intact controls	1	22.0	98.7	325	37.7	42.8	50.5
	2	21.0	126.2	1060	38.8	43.0	53.9
	3*	21.0	101.6	540	39.3	60.8	42.2
	4*	21.0	113.0	560	38.5	71.5	48.9
Mean and Standard Error		21.2±0.25	109.8±6.2	621±155	38.6±0.34	54.5±7.1	48.9±2.4
Sham-operated Lt.	5*	15.5	85.0	200	36.4	45.2	53.7
Rt.	6	20.5	86.0	310	35.1	23.5	33.0
Rt.	7	23.5	130.0	250	40.3	21.4	41.0
Lt.	8	25.0	135.0	720	37.5	71.0	45.3
Mean and Standard Error		21.1±2.1	109.0±13.6	370±118	37.3±1.12	40.2±11.7	43.3±4.3
Left Atrial Stretch	9*	20.0	103.5	220	36.5	23.7	36.8
	10	22.5	107.7	100	37.3	20.6	60.5
	11*	23.0	92.7	390	35.5	18.5	50.5
	12	21.5	110.0	370	36.4	19.9	25.5
	13*	20.0	104.0	400	36.6	35.5	38.5
Mean and Standard Error		21.4±0.62	103.5±3.0	296±59	36.4±0.29‡	23.6±3.1‡	42.3±6.0
Right Atrial Stretch	14	26.5	91.1	—	35.8	36.7	11.9
	15*	21.5	95.0	320	37.2	18.4	26.8
	16*	21.5	125.0	350	39.3	16.8	30.7
	17*	22.0	77.1	200	37.9	6.8	29.4
	18	21.0	120.5	400	38.9	20.8	20.4
Mean and Standard Error		22.5±1.0	101.7±9.2	318±42	37.8±0.63	19.9±4.8‡	23.8±3.5†

* Infusion of norepinephrine in 5% glucose solution was necessary during some part of the experimental period.
† These values are significantly different from those of the sham-operated group ($p < .01$).
‡ See text for discussion of statistical validity.

norepinephrine is without effect on hydrocortisone and aldosterone secretion in the cat. The average arterial blood pressure during the period of adrenal venous blood collection was not significantly different among the various experimental groups (Table 1).

Venous pressure was measured in the external jugular vein in two additional animals by means of a saline manometer adjusted to zero at the level of the right atrium. In both animals an increase of approximately 5 cm. saline pressure was noted when the chest was opened. In one animal an additional increase of 2 cm. saline pressure was seen when the pericardium was elevated. In both animals stretching of the right atrium resulted in a decrease of 0.5 cm. saline pressure. It seems probable that the modest change in venous pressure following right atrial stretch would not have a significant effect on the experimental results, although further study is clearly needed.

The volumes of adrenal venous blood collected over the two hour bleeding periods are presented in Table 1. The average volumes obtained from the three groups of animals subjected to open chest surgery were very similar and appeared to be somewhat less than in the intact group. However, large variations were noted, and the differences between the means are not satistically significant. The higher average volume of adrenal venous blood in the intact group appears to be due to an unusually large adrenal blood flow in one animal (Table 1). Careful examination of the data failed to reveal any consistent correlation between volume of adrenal venous blood and steroid output. This is in agreement with earlier observations that the steroid secretory rate is largely independent of the adrenal blood flow within wide limits (7).

The body temperatures tended to fall during the course of the experiments. However, the average body temperatures in the open-chest sham and right atrial stretch groups, though slightly lower, are not significantly different from those of the intact controls. On the other hand, the left atrial group had a significantly lowered temperature, as compared to the intact controls. Whether this reduction (about 2.2° C) affected the results with respect to hydrocortisone secretion remains unanswered. The work of Egdahl, Nelson and Hume (14) indicates that hydrocortisone secretion is reduced in hypothermic animals, although the temperature ranges at which marked reductions in hydrocortisone secretion were observed in their experiments were much lower than are reported here. In any event, the reduction in body temperature in the left atrial stretch group was apparently without effect on the aldosterone secretion rate, since the rates of output of this steroid were within the control range.

The rates of secretion of hydrocortisone were somewhat variable. In the sham-operated group the rate of output of hydrocortisone seemed to be on the average somewhat less than in the intact control group. However, because of variation among the individual animals this apparent difference

is not statistically significant. The values in the right and left atrial stretch groups are significantly lower than in the intact controls ($p < .01$) but not when compared to the more appropriate controls, that is, the sham-operated group ($P < .2 > .1$). Interpretation of the data is consequently difficult, and a final statement must await further studies using larger numbers of animals. It seems likely that if the number of animals in the sham-operated group were increased, a significant depressing effect of the open-chest procedure *per se* could be demonstrated, as well as a further depression by atrial stretch.

The rates of secretion of aldosterone are presented in Table 1. In the control animals the aldosterone secretion rate was 48.9 ± 2.45 (S.E.) μg./100 kg. body wt./hr. In the four sham-operated animals the aldosterone secretion rate was 43.3 ± 4.30. This value is not significantly different from that in the controls ($P < 0.3 > 0.2$). When the left atrium was stretched the rate of secretion of aldosterone was 42.2 ± 6.03, which is not significantly different from that in either the sham-operated or the intact control group ($P < 0.4 > 0.3$). In the dogs subjected to right atrial stretch the aldosterone secretion rate was 23.8 ± 3.46 μg./100 kg. body wt./hr. This is a highly significant reduction in secretion rate as compared to the intact ($P < .01$), sham-operated ($P < .01$), and left atrial stretch groups ($P < .05$).

Distention of the right and left atrium by external tension was chosen in favor of a balloon in the atrium as used by Henry and co-workers because it was felt that the former would permit direct visualization of the degree of distention and that it would not impede blood flow through the heart. Cardiac output determinations were not carried out in these experiments. The decrease in aldosterone secretion that was seen following stretching of the right atrium may have been mediated by a decrease in blood flow through a sensitive area on the arterial side of the vascular system, or by congestion on the venous side. If this is the case, it appears that the effects on these parameters of right atrial stretch are much different from those of left atrial stretch, since the aldosterone secretion rate was significantly different between the two groups. It is of interest that the adrenal blood flow was approximately equal in these two experimental groups, indicating that if a difference in cardiac output was actually present, it was not reflected in a change in adrenal blood flow.

The existence of nerve endings in the atria, bases of the venae cavae and pulmonary veins which could serve as stretch receptors has been documented histologically by Nonidez (15). Paintal has recorded impulses in vagal fibers which seem to respond to changes in atrial filling (16). Although it seems a reasonable possibility, it remains to be demonstrated that these receptors are concerned in the reflex suppression of steroid secretion suggested by our experiments.

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REFERENCES

1. Peters, J. P.: Diseases of Metabolism, 3rd edition, G. G. Duncan, ed., p. 363, W. B. Saunders, New York, 1952.
2. Smith, H. W.: *Am. J. Med.* **23**: 623. 1957.
3. Gaunt, R. and J. J. Chart: *Symposium on Homeostatic Mechanisms*, Brookhaven National Laboratory, June 1957.
4. Falbriard, A., A. F. Muller, J. Crabbe and A. Duckert-Maultshech: *Helv. Med. Acta.* **22**: 495. 1955.
5. Beck, J. C., I. Dyrenfurth, C. J. P. Giroud and E. H. Venning: *Trans. Assn. Am. Physicians*, **68**: 205, 1955.
6. Bartter, F. C., G. W. Liddle, L. E. Duncan, Jr., J. K. Barker and C. Delea: *J. Clin. Invest.* **35**: 1306. 1956.
7. Farrell, G. L., R. S. Rosnagle and E. W. Rauschkolb: *Circ. Res.* **4**: 606. 1956.
8. Goodkind, M. J., W. C. Ball and J. O. Davis: *Am. J. Physiol.* **189**: 181. 1957.
9. Leutscher, J. A., Jr.: *Recent Prog. Hormone Res.* **12**: 175. 1956.
10. Henry, J. P., O. H. Gauer and J. Reeves: *Circ. Res.* **4**: 85. 1956.
11. Sweat, M. L. and G. L. Farrell: *Proc. Soc. Exper. Biol. and Med.* **87**: 615. 1954.
12. Farrell, G. L., P. C. Royce, E. W. Rauschkolb and H. Hirschman: *Proc. Soc. Exper. Biol. and Med.* **87**: 141. 1954.
13. Newman, A. E., E. S. Redgate, F. M. Yatsu and G. L. Farrell: *Fed. Proc.* April 1958 (in press).
14. Egdahl, R. H., D. H. Nelson and D. M. Hume: *Surg. Gynec. and Obst.* **101**: 715. 1955.
15. Nonidez, J. F.: *Am. J. Anat.* **61**: 203, 1937.
16. Paintal, A. S.: *J. Physiol.* **120**: 596. 1953.

IN VITRO METABOLISM OF TRIIODOTHYRONINE[1]

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ABSTRACT

Previous studies have shown that rat kidney slices contain an enzyme which deiodinates thyroxine to yield triiodothyronine. Further, a soluble enzyme has been obtained from sonically disintegrated rat kidney mitochondria which converts thyroxine and triiodothyronine to the corresponding acetic acid analogues.

In the present study the metabolism of I^{131}-labelled triiodothyronine by intact and solubilized mitochondria of rat kidney, liver, heart and brain and by homogenates of spleen and intestine was examined.

With the intact mitochondrial enzyme system two types of reactions were observed. Deiodination of triiodothyronine occurred most actively in kidney, liver, heart, spleen and brain. Intestine showed very little deiodination. The second reaction involved the conversion of triiodothyronine to triiodothyroacetic acid. This was observed chiefly in kidney, although a small peak of triiodothyroacetic acid radioactivity was regularly observed with liver.

Sonic disruption of the mitochondria resulted in loss of the deiodinating activity and an increase in the conversion of triiodothyronine to triiodothyroacetic acid by kidney, liver, heart and brain.

The presence of the enzyme system for converting triiodothyronine to triiodothyroacetic acid in the mitochondria of organs other than the kidney increases the likelihood that it has a physiologic role in the action of the hormone. In this connection it should be noted that the tissues most susceptible to the stimulating effect of thyroid hormone (heart, kidney, liver) are the most active in this transformation.

THYROXINE is metabolized in several ways by extrathyroidal tissue. Some of these, such as the conjugation with glucuronic acid and disruption of phenoxy linkage (1) by the liver, are presumably detoxicative. The deiodination of thyroxine to triiodothyronine, on the other hand, might be prerequisite to the peripheral action of the thyroid hormone. The best evidence for this hypothesis was given by Sheehan (2), MacLagan (3) and Wilkinson (4) who demonstrated that n-butyl 4-hydroxy-3:5-diiodobenzoate which blocks the deiodination of both thyroxine and triiodothyronine, suppresses the metabolic effect of the former while enhancing the

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latter. The conversion of thyroxine to triiodothyronine has been observed repeatedly in thyroidectomized animals and in *in vitro* systems.

Further reactions involving the alanine side chain of both thyroxine and triiodothyronine have been reported. Conversion of thyroxine and triiodothyronine to their corresponding acetic acid analogues by cell free enzyme systems prepared from rat kidney mitochondria was observed (5, 6). A similar reaction has been found in the case of triiodothyronine in the intact animal (7, 8). It is not known whether such a transformation is degradative or important to the derivation of an intracellularly active hormone. Support has been given to the latter alternative by the observation that triiodothyroacetic acid is approximately 10 times more active than triiodothyronine in uncoupling oxidative phosphorylation in heart mitochondria (9), a proposed mode of action of the thyroid hormone.

If triiodothyroacetic acid is a metabolically active derivative of triiodothyronine it is probably formed *in situ* since it has been shown that following intravenous administration in the intact animal it is rapidly eliminated by the liver (10). In the original studies only kidney was used; therefore, it is important to examine other tissues for this specific enzyme system. The present paper presents experiments on the *in vitro* metabolism of triiodothyronine by various organs including brain, heart, liver, spleen, intestine and kidney.

METHODS

Materials and Preparation

Organs of female rats (Sprague-Dawley 150–200 gm.) were used in all experiments. After homogenization in sucrose the mitochondria of kidney, liver, heart and brain were isolated by the method of Schneider (11). Because of difficulty in the isolation of mitochondria from spleen and intestine, whole homogenates centrifuged for ten minutes at 4° C at 600 G were used. The soluble enzyme systems were prepared by disruption of the mitochondria of kidney, liver, heart and brain in the Raytheon 10 KC sonic oscillator for eight minutes with alcohol at −5° C as the coolant. The sonated preparation was centrifuged at 100,000 G for 30 minutes and the supernatant dialyzed against 2 liters of distilled water at 4° C for 4 hours.

Reaction Procedure

The reaction mixtures (final volume 3 ml.) contained 2.5 μ moles of DPN, 50 μ moles of phosphate buffer, pH 7.4, and 2 ml. of enzyme preparation. (Enzyme concentration: homogenate 10%; intact mitochondria 20–25 mg. of protein; solubilized mitochondria 5–10 mg. protein except for heart which was 1–3 mg. protein). To each flask was added .05 micrograms of I^{131}-labelled chromatographically pure l-triiodothyronine.[2] The reaction mixtures were incubated at 37° C for 2 hours. Enzyme preparation boiled for 10 minutes and unincubated systems were used as controls.

Separation of Products

At the end of incubation a 150 lambda aliquot of the reaction mixture was chromato-

[2] Obtained from Abbott Laboratories.

graphed directly. Fifty micrograms of non-radioactive triiodothyronine,[3] triiodothyroacetic acid[4] and iodide were added to the mixture as markers before application to the paper. The solvent system employed was tertiary amyl alcohol saturated with 2N NH_4OH run in descent for 24 hours at room temperature (6). The radioactivity was located in the strips by a continuously recording chromatogram scanner equipped with an end-window G.M. counter. The position of the thyronine marker compounds was determined by color development with 4-amino-antipyrine reagent (12), and iodide by a starch-iodate reagent.

RESULTS

With the intact mitochondrial enzyme system prepared from the organs studied two types of reactions were observed. As shown in Figure 1, deiodination of triiodothyronine occurred most actively in kidney and liver, with less activity in heart, spleen and brain. Intestine showed very little deiodination. The second reaction involved the conversion of triiodothyronine to triiodothyroacetic acid. As seen in Figure 1, this was observed chiefly in kidney, although a small peak of triiodothyroacetic acid radioactivity was regularly observed with liver.

With the solubilized mitochondrial enzyme systems the deiodination of triiodothyronine was sharply reduced. As shown in Figure 2, disruption of the mitochondria resulted in the loss of deiodinating activity in kidney, liver, heart, and brain. In contrast, the conversion of triiodothyronine to triiodothyroacetic acid appeared to be enhanced by sonic disintegration of the mitochondria. Comparison of Figure 1 and Figure 2 reveals the increased formation of triiodothyroacetic acid by solubilized mitochondria of kidney and liver, and the formation of a detectable quantity of triiodothyroacetic acid by the heart preparation. Brain activity remained equivocal.

DISCUSSION

Deiodination reactions involving thyroxine have been observed in a variety of tissue preparations. In rat kidney slices the major deiodination product was triiodothyronine (13, 14). Homogenates and extracts of several tissues have been studied by MacLagan (15) and Ruegamer and Yunis (16). With these preparations the deiodination proceeded more rapidly and the formation of triiodothyronine was not observed. Triiodothyronine was deiodinated apparently by the same enzyme system at a rate at least equal to the deiodination of thyroxine. Ruegamer and Yunis observed further that the deiodinating activity of their homogenate preparations was associated with the particulate matter of the cells. The present study indicates that in the case of triiodothyronine the deiodinase enzyme exists in connection with the mitochondria of kidney, liver, heart, spleen and brain. Further, this activity is dependent upon the integrity of the

[3] Obtained from Smith, Kline and French Laboratories through the courtesy of A. E. Heming.

[4] Obtained from Hoffmann-La Roche, Inc. through the courtesy of M. J. Schiffrin.

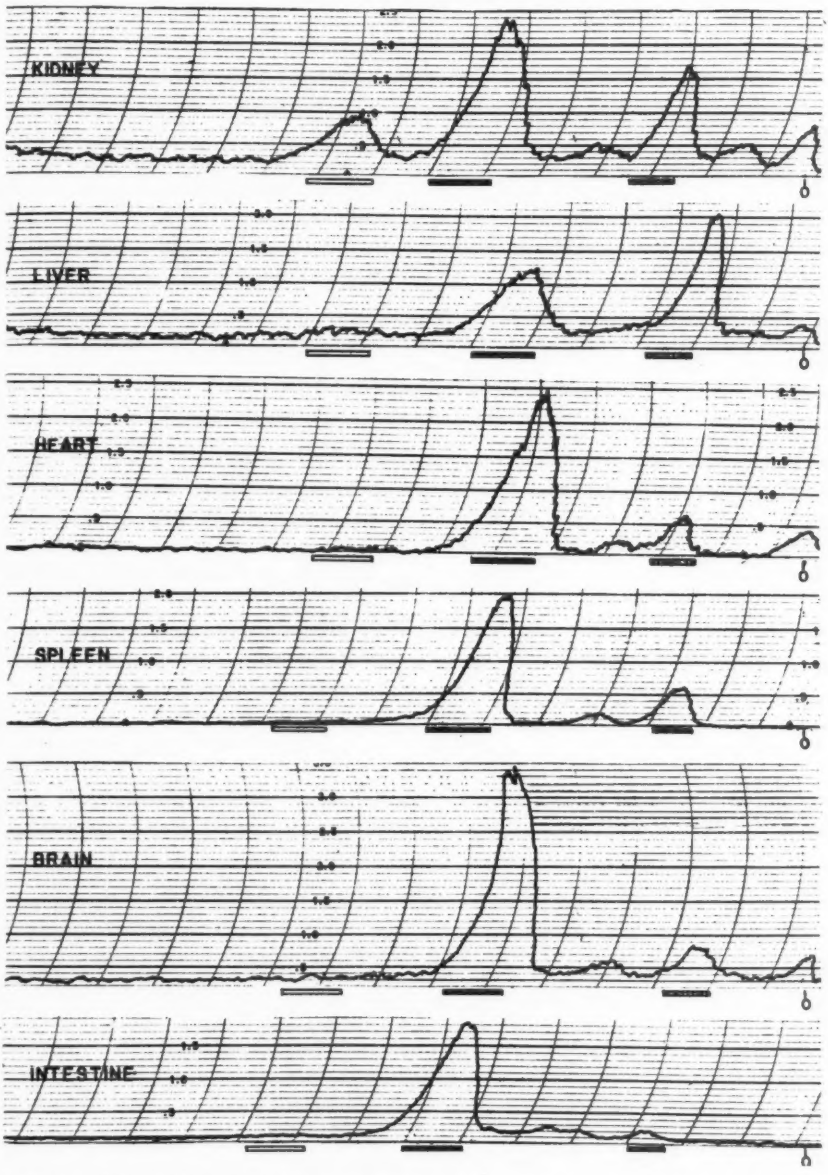

FIG. 1. Metabolism of labelled triiodothyronine by intact mitochondria of kidney, liver, heart and brain, and by homogenate of spleen and intestine. Position of non-radioactive marker iodide shown by cross hatched bar, triiodothyronine by solid bar, and triiodothyroacetic acid by open bar. O—origin of chromatogram. Chromatographic front not shown.

mitochondria as deiodination is sharply reduced following sonic disruption of these particles. The loss of deiodinating activity attendant upon disruption of the mitochondria is believed not to be due to dilution effect, as was reported by Tata (17) working with dog brain suspension, since the same weight of tissue was used in preparation of both intact and solubilized mitochondria. Failure to demonstrate deiodination in previous experiments with intact mitochondria was probably due to the extraction of the reaction mixture with butanol prior to chromatography.

If the deiodinating enzyme observed in the slice experiments and in the cell free preparations is the same, the accumulation of triiodothyronine in the intact cell suggests the presence of a trapping system which is de-

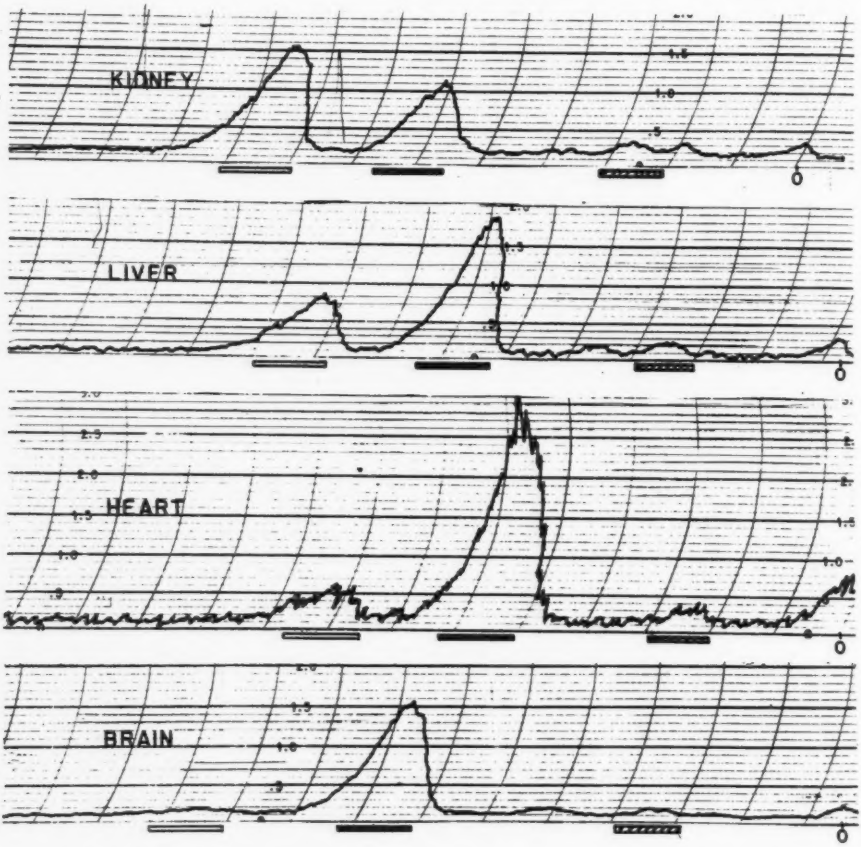

FIG. 2. Metabolism of labelled triiodothyronine by solubilized mitochondrial enzyme system of kidney, liver, heart and brain. Position of non-radioactive marker iodide shown by cross hatched bar, triiodothyronine by solid bar, and triiodothyroacetic acid by open bar. O—origin of chromatogram. Chromatographic front not shown.

stroyed by the disruption of the cell. Within this hypothesis, such a system might provide a self regulatory mechanism within the cell. The accumulation of triiodothyronine would enhance the metabolic activity of the hormone, either *per se* or as a precursor of an active compound derived possibly through alterations in the alanine side chain. Deiodination of triiodothyronine, on the other hand, would result in loss of hormonal activity.

The enzymatic formation of triiodothyroacetic acid from triiodothyronine was first observed using rat kidney mitochondria (5). A preliminary survey of other organs of the rat failed to reveal similar activity. The use of intact mitochondria or homogenates in the initial survey probably accounted for the failure to observe triiodothyroacetic acid formation. It is clear from the present study that disruption of the mitochondria enhances the triiodothyronine to triiodothyroacetic acid conversion. This may be accounted for at least in part by the destruction of the deiodinating system by sonic disruption, which in intact mitochondria competes for the labelled substrate. In liver, where deiodinating activity was the greatest, for example, it was difficult to demonstrate triiodothyroacetic acid formation with intact mitochondria, whereas with the solubilized enzyme rather large amounts of triiodothyroacetic acid were produced.

The presence of this enzyme system in the mitochondria of tissues other than kidney, together with the observation that intravascular triiodothyroacetic acid is rapidly eliminated (10), makes it appear unlikely that triiodothyroacetic acid is formed in the kidney for use elsewhere in the body. It appears more likely that many tissues can carry out this conversion. The presence of this enzyme system in these tissues increases the likelihood that it has a role in the metabolism of the hormone. That this role may be physiologic is suggested by the observation that tissues most susceptible to the stimulating effect of thyroid hormone (heart, kidney and liver) (18), are most active in converting triiodothyronine to triiodothyroacetic acid. It must be emphasized that the presence of triiodothyroacetic acid in this metabolic sequence does not necessarily mean that it is the active hormone, since it may well be a precursor or product or one of many hormones.

Acknowledgment

The assistance of Miss Sue Ames, Medical Technician, is gratefully acknowledged.

REFERENCES

1. Plaskett, L. G.: *Nature* **181**: 273, 1958.
2. Sheehan, M. M., J. H. Wilkinson and N. F. MacLagan: *Biochem. Jour.* **48**: 188. 1951.
3. MacLagan, N. F., W. E. Sprott and J. H. Wilkinson: *Lancet* **2**: 915. 1952.
4. Wilkinson, J. H., W. E. Sprott, C. H. Bowdon and N. F. MacLagan: *Biochem. Jour.* **56**: 215. 1954.
5. Albright, E. C., F. C. Larson, K. Tomita and H. A. Lardy: *J. Clin. Invest.* **35**: 687. 1956, and *Endocrinology* **59**: 252. 1956.

6. Tomita, K., H. A. Lardy, F. C. Larson and E. C. Albright: *J. Biol. Chem.* **224**: 387. 1957.
7. Roche, J., R. Michel, P. Jouan and W. Wolf: *Endocrinology* **59**: 425, 1956.
8. Roche, J., R. Michel and P. Jouan: *Comp. Rendu Soc. Biol.* **150**: 629. 1956.
9. Wang, S. and H. A. Lardy. Personal communication.
10. Larson, F. C. and E. C. Albright: *Endocrinology* **63**: 183. 1958.
11. Schneider, W. C. in Umbreit, W. W., Burris, R. H. and Stauffer, J. F.: Manometric Techniques and Tissue Metabolism, Minneapolis, 153. 1951.
12. Emerson, E.: *J. Organic. Chem.* **8**: 417. 1943.
13. Albright, E. C., F. C. Larson and R. H. Tust: *Proc. Soc. Exp. Biol. and Med.* **86**: 137. 1954.
14. Larson, F. C., K. Tomita and E. C. Albright: *Endocrinology* **57**: 338. 1955.
15. MacLagan, N. F. and D. Reid: *Ciba Foundation Colloquia on Endocrinology* **10**: 190. 1957.
16. Ruegamer, W. R., and S. Yunis: *Fed. Proc.* **16**: 239. 1957.
17. Tata, J. R., J. E. Rall and R. W. Rawson: *Endocrinology* **60**: 83. 1957.
18. Barker, S. B.: *Endocrinology* **59**: 548. 1956.

PATHOLOGIC CHANGES IN ADRENALECTOMIZED, SENSITIZED RATS TREATED WITH ADRENAL CORTEX EXTRACT[1]

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ABSTRACT

Male rats of the Sprague-Dawley strain were adrenalectomized and unilaterally nephrectomized at an initial weight of about 300 gms. The rats were adapted to the tube-feeding of a medium carbohydrate diet which contained 4% added NaCl by dry weight. The following doses of adrenal cortex extract (ACE) were given by subcutaneous injection for 56 days: 0.0, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 cc. per rat per day. When the dose of ACE was 1.0 cc. per day or greater the blood pressure was elevated in proportion to the dose. Doses of 4 and 8 cc. of ACE per rat per day caused an increase in the amount of renal and myocardial pathology. Arteriosclerosis was not seen in any animal of the present study but has been seen in a small number of sensitized rats given large doses of ACE in other experiments.

A NUMBER of sodium-retaining synthetic steroids cause damage to the kidney and to the cardio-vascular system when administered to experimental animals in high dosage. Damage is more likely to result when the animal is sensitizied by the removal of one kidney and by the administration of a high load of sodium chloride (1). Ingle and Baker (2) observed renal and cardiovascular damage in sensitized rats given large doses of adrenal cortex extract (ACE) and in subsequent experiments (3, 4) we have observed some elevation in blood pressure and some pathologic changes when doses of ACE nearer the physiological range were given to the sensitized rat.

This is a report on the dose-response effect of ACE upon blood pressure and tissue changes in the adrenalectomized-sensitized rat. The data show a significant relationship between the dose of ACE and the extent of the changes from normal.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of approximately 300 grams. They were then adapted to the tube-feeding of a fluid high sodium chloride diet (Table 1) in the amount of 13 cc. each morning and late afternoon. Following adaptation to the diet all of the rats were subjected to unilateral nephrectomy plus bilateral adrenalectomy. Clean but not sterile

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technique was used. Immediately following operation each rat was given 5000 units of penicillin and 5 mgm. of streptomycin. This was not continued. Some of the rats were kept without replacement therapy; the others were given varying doses of beef adrenal cortex extract (Upjohn), 1 unit per ml., in aqueous solution (ethanol-free) by subcutaneous injection twice daily for a period of 56 days.

For the determination of blood pressure the rats were anesthetized with ether and the abdominal aorta was cannulated below the level of the renal arteries. The cannula was attached by a short length of fine-bore rubber tubing filled with heparinized saline to a mercury manometer. The record was taken when anesthesia was light, as judged by retraction of the rat's hind foot on pinching.

All rats were killed by exsanguination under ether anesthesia at the end of 8 weeks. Autopsy was performed and gross changes noted. The heart, kidney and portions of liver, spleen, pancreas, lung, stomach and intestine were fixed in 10% neutral formalin.

TABLE 1. HIGH SALT, MEDIUM CARBOHYDRATE DIET.

Constituent	Grams
Cellu flour	60
Salt mixture U.S.P. XIV	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil plus 100 mg. K	10
Mazola oil	190
Casein	160
Starch	300
Dextrin	150
Sucrose	140
Sodium chloride	50

Hematoxylin and eosin was the routine stain and selected examples of heart and kidney were examined by the periodic acid-Schiff routine, and Weigert's resorcin-fuchsin stain for elastic tissue.

EXPERIMENTS AND RESULTS

ACE was administered in amounts ranging from 0.1 cc. per rat per day to 8 cc. per rat per day. Dosage in relation to results is shown in Table 2. Eleven adrenalectomized-sensitized rats were kept without ACE; the high salt diet supported life.

Survival. One rat died with symptoms of adrenal cortical insufficiency. Five rats died after becoming completely anuric. The body cavities were filled with fluid. The ureter of each of the 5 rats was found to be obstructed by gravel, the kidney of each had developed some degree of hydronephrosis and the pelvis contained gravel. We have no insight into the reasons for formation of the kidney stones but noted that death occurred only in the rats which were either treated with small doses of ACE or were untreated.

Growth. The higher doses of ACE caused some suppression of the amount of gain in weight and there was some suppression of hair-growth. The animals treated with moderate doses of ACE gained more weight than the untreated animals. There was some atrophy of thymus when the dose of ACE

was large and an increase in the size of thymus when the dose of ACE was small or when none was given.

Blood pressure. The blood pressure was elevated above the average for untreated rats when the dose of ACE was 1 cc. or greater per rat per day: the extent of increase was related to the size of the dose. The average values for all of the sensitized rats of this study was higher than the average value for normal rats.

TABLE 2. EFFECT OF ACE IN ADRENALECTOMIZED-SENSITIZED (UNILATERAL NEPHRECTOMY, 4% NaCl DIET) FOR 56 DAYS. TUBE-FEED 13 CC. 2×DAY. AVERAGES AND STANDARD ERRORS

Experimental conditions	No. rats	Gms. body weight		Organ wts., mgm.			Mm. Hg BP	Ratings of pathology*		
		Initial	Final	Heart	Kidney	Thymus		Kidney		Heart
								Gross	Micro	Micro
8 cc. ACE day	6	311 ±4.0	381 ±4.6	1370 ±28.3	2358 ±91.2	220 ±16.9	181 ±7.5	2.7 ±0.4	2.67 ±0.41	1.0 ±0.22
4 cc. ACE day	6	313 ±2.6	393 ±3.9	1380 ±28.6	2245 ±82.4	290 ±18.5	159 ±5.0	2.3 ±0.4	2.50 ±0.37	0.67 ±0.20
2 cc. ACE day	6	319 ±3.6	415 ±8.8	1313 ±31.1	2025 ±90.1	549 ±79.8	146 ±5.9	0.7 ±0.02	1.08 ±0.26	1.30 ±0.25
1 cc. ACE day	7	313 ±4.4	397 ±5.3	1197 ±40.5	1839 ±43.0	543 ±48.7	139 ±1.0	0.6 ±0.01	2.00 ±0.51	0.28
0.5 ACE day	4	313 ±1.3	418 ±4.2	1245 ±30.7	2077 ±46.4	634 ±87.3	128 ±4.5	0.12 ±0.01	1.25 ±0.20	0.00
0.2 ACE day	4	321 ±4.2	430 ±4.9	1267 ±18.4	2002 ±58.1	739 ±60.5	130 ±3.2	0.38 ±0.02	0.50 ±0.19	0.00
0.1 ACE day	6	319 ±3.5	412 ±5.0	1195 ±36.6	1885 ±90.1	676 ±45.1	129 ±1.4	0.58 ±0.08	0.50 ±0.21	0.33
No ACE	11	316 ±2.9	402 ±4.9	1154 ±16.9	1767 ±40.8	658 ±40.8	131 ±1.7	0.73 ±0.17	0.91 ±0.27	0.18
Normals low salt diet	6	308 ±2.5	408 ±3.8	1130 ±20.2	1335 ±25.4	462 ±34.1	122 ±1.3	0.0		

* The ratings of gross renal pathology are based on the following scale: 0 = no abnormality; 1 = few tiny nodules; 2 = moderately nodular with some mottling; 3 = more nodules, mottling with some gray spots; 4 = rough, mottled, with gray and red spots; and 5 = changes listed previously more severe, giving "flea-bitten" grayish-yellow appearance.
The ratings of the microscopic changes are based upon the following features: KIDNEY: 0 = no abnormality; 1 = hypertrophy of glomeruli and tubules, a few hyaline casts, and slight focal interstitial inflammation; 2 = active pelvic inflammation; 3 = glomerular lesions, no apparent vascular damage; 4 = hyalinization of glomeruli, sclerosis of arterioles; and 5 = necrosis of glomeruli and arterioles. HEART: 0 = no abnormality; 1 = hypertrophy of muscle; 2 = focal inflammation or fibrosis of myocardium; 3 = changes listed previously, sclerosis of vessel walls; and 4 = necrosis of myocardium and/or vessel walls.

Heart. The average weight of the heart was greater in the rats given large doses of ACE. One rat given 8 cc. of ACE daily had visible gray spots in the wall of the heart.

As in previous experiments (3, 4, 5), the microscopic changes in the heart were assessed on the basis of muscle hypertrophy (grade 1), the presence of granulomatous or fibrotic foci in the myocardium (grade 2), and hypertensive damage to the coronary blood vessels (grades 3 and 4; footnote to Table 2). Changes in the heart were more common in the rats treated with 8, 4 and 2 cc. of ACE (Table 2). The cardiac muscle cells hypertrophied and in some rats this was associated with focal lesions of the myocardium. They consisted of collections of macrophage cells, Anitschkow myocytes, and fibroblasts within the interstitial tissue (Fig. 1). Other rats showed focal myocardial fibrosis, sometimes in relation to branches of the coronary

arteries. However, despite the record of hypertension obtained with the higher levels of ACE administration there was no evidence of hypertensive vascular damage to the arteries of the heart in any rat.

Kidney. There was a positive relationship between the dose of ACE and the average weight of the kidney. At doses of 4 and 8 cc. of ACE per rat per day there was an increase in the amount of gross damage consisting in mottling, roughness due to pits and nodules, and some kidneys had gray spots and red spots. At doses of 2 cc. and less the damage was minimal.

As in previous experiments (3, 4, 5) the degree of microscopic damage to the kidney (footnote, Table 2) was assessed on the basis of focal inflam-

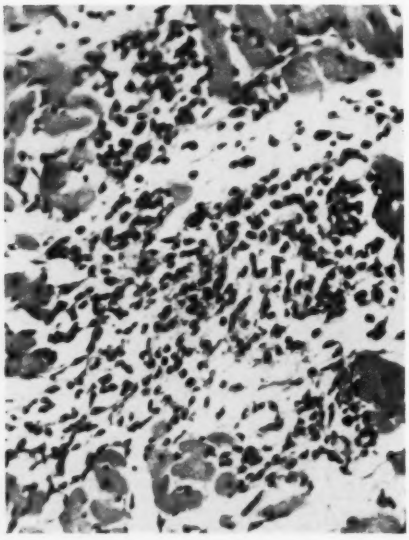

FIG. 1. Heart. ACE 8 cc. daily. The cardiac muscle fibres are separated by a focus of fibroblasts and macrophage type cells. B.P. 195 mm. Hg. Haematoxylin and eosin ×225.

mation in the cortex or pelvis (grades 1, 2), glomerular tuft lesions (grade 3), and the association of glomerular damage with hypertensive changes in the renal arterioles (grades 4, 5). Small focal areas within the cortex showing periglomerular fibrosis, a few casts with atrophy of tubular epithelium, and some round cell infiltration were present in some rats in all the experimental groups. Active chronic inflammation of the renal pelvis was seen in 9% of the rats, but this was not confined to animals of any one group. Glomerular tuft lesions occurred in rats treated with 8, 4 or 2 cc. of ACE (Table 2), but examples of this type of damage were seen in 2 rats maintained on the salt diet without hormone. The glomerular lesions consisted of an increase in intercapillary hyaline material. In some instances the tuft was shrunken and the subcapsular space enlarged, while in others the hyalinized tuft adhered to a thickened Bowman's capsule so obliterating the space (Figs. 2 & 3). Although the blood pressure in rats treated with higher doses of ACE was elevated there was no evidence in

FIG. 2. Kidney. ACE 4 cc. daily. The glomerulus is partly hyalinized and adherent to Bowman's capsule. The tubules are dilated. B.P. 158 mm. Hg. H.&E. ×185.

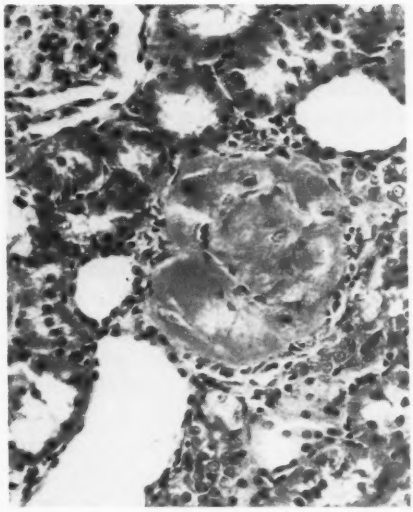

FIG. 3. Kidney. ACE 4 cc. daily. The glomerulus is almost completely sclerosed and the subcapsular space is obliterated. The tubules are dilated. B.P. 150 mm. Hg. H.&E. ×250.

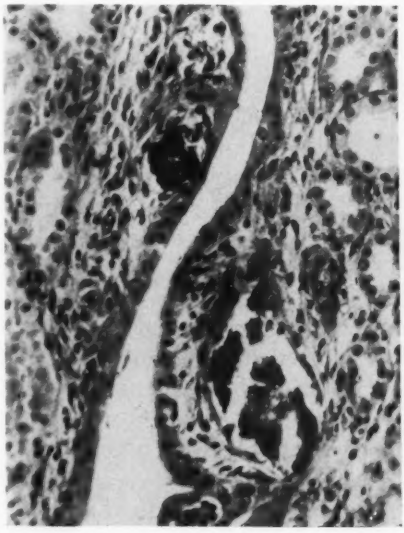

FIG. 4. Kidney. ACE 4 cc. daily. Deposits of calcified debris are present beneath the epithelium of the renal pelvis. H.&E. ×270.

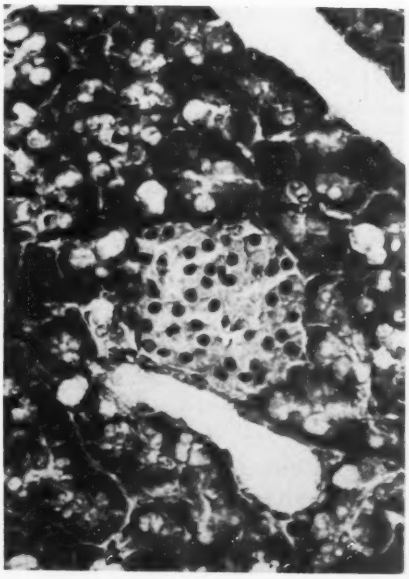

FIG. 5. Pancreas. ACE 8 cc. daily. "Signet-ring" cell change is present in the acinar tissue. H.&E. ×295.

these experiments of hypertensive arteriolosclerosis or arteriolonecrosis affecting the arterioles of the kidney. In view of the deaths at the beginning of these experiments due to renal stone and hydronephrosis, it is of interest to record that 9 of the rats killed at the end of the investigation showed deposits of calcium salts beneath the epithelium of the renal pelvis (Fig. 4). Examples were seen in animals treated with ACE at several dose levels, including the higher range, and also in one rat maintained only on the diet.

Other organs. Hypertensive vascular lesions were absent in the other organs taken for examination. Eight rats showed a focal "signet-ring cell" change in the pancreatic acinar tissue (Fig. 5). The significance of this finding is not clear, but it has been described by Salgado and Selye (6) in the rat following treatment with methylandrostenediol. In our experiments it occurred in rats treated with ACE and in 1 rat maintained solely on the salt diet.

DISCUSSION

We anticipated some similarity in the kinds of tissue damage in this and in related studies (3, 4, 5). Unlike our previous results we failed to reproduce hypertensive arteriosclerosis, although hypertensive records were obtained manometrically in the present experiment. The rise in blood pressure and the extent of pathology showed a positive relationship to the dose of adrenal cortical extract. Untreated sensitized rats showed an average blood pressure higher than normal average and there was more microscopic damage to hearts and kidneys than occurs in normal animals on a normal intake of sodium.

It may be appropriate here to discuss more fully the relationship of the pathological findings in the kidney to the rise in blood pressure. The most common finding in animals of all groups was foci of dilated tubules containing protein casts and lined by a rather flattened epithelium with basophilic cytoplasm. In many cases the related glomerulus showed periglomerular fibrosis and an increase of lymphocytes was present in the adjacent interstitial tissue (grade 1 lesion). Such changes have been described in the kidney of the aged rat by Ingle and Baker (2) and more recently by Kennedy (7). The latter has shown that they develop at a rather earlier age, between one year and 18 months, in the unilaterally nephrectomized rat. Using much younger rats we have considered that these appearances are a result of focal chronic pyelonephritis although the mechanism of their production may be more intricate. They are not necessarily associated with obvious ascending infection in the medullary tubules.

Glomerulosclerosis is also seen in the aging rat. In the present experiments it occurred most frequently in those rats treated with the higher doses of adrenal cortex extract in which hypertensive records were obtained, but hypertensive arteriosclerosis was not seen in any animal. We have no firm conviction that glomerulosclerosis alone (grade 3 lesion) can

be regarded as hypertensive in nature in the rat. Hypertensive vascular damage has been seen in a few individual sensitized rats given ACE in earlier experiments (3, 4). Here the rats were allowed to eat the 4% sodium chloride diet *ad libitum*, but the daily intake of the group was measured. The actual dietary intake of individual rats was not controlled.

Small doses of ACE, as little as 1 cc. per rat per day, cause an elevation of blood pressure in the sensitized-adrenalectomized rat. Rats given 1 and 2 cc. of ACE per rat per day have thymus glands which are heavier than average, a sign of insufficiency of thymolytic steroid in this dosage of ACE. Elsewhere (3, 4) we have noted that the non-adrenalectomized rat given a high salt load may suppress its secretion of sodium-retaining steroids without a concomitant decrease in secretion of glucocorticoids. The adrenalectomized rat given a constant intake of ACE is at a disadvantage in dealing with a high load of salt; an excess of sodium-retaining steroid may coexist with a relative deficiency of glucocorticoids.

It is tempting to compare the effects of ACE in the sensitized rat with the effects of adrenal enucleation in the sensitized rat, but it is not shown that the administration of ACE can fully simulate the effects of adrenal enucleation which include severe hypertension and arteriosclerosis. The regenerating enucleated adrenal has not been found to secrete a greater than normal amount of sodium-retaining steroid (8). It is conceivable that the regenerating adrenal causes trouble by failing to suppress its secretion of sodium-retaining steroid to a minimum as can the normal adrenal which adjusts to a high salt load. Studies of the pattern and amounts of steroid secreted by the regenerating adrenal should include a time-response comparison of steroid output by normal and regenerating adrenals at both high and low salt loads.

REFERENCES

1. Selye, H. and G. Heuser. Fifth Annual Report on Stress. MD Publications, Inc. New York, 1956.
2. Ingle, D. J. and B. L. Baker. *Recent Progress in Hormone Research*. **8**: 143. 1953. New York, Academic Press, Inc.
3. Crane, W. A. J., B. L. Baker and D. J. Ingle. *Endocrinology* **62**: 216. 1958.
4. Crane, W. A. J., R. E. Porter and D. J. Ingle. *Endocrinology* **63**: 43. 1958.
5. Crane, W. A. J. and D. J. Ingle. *Endocrinology* **62**: 474. 1958.
6. Salgado, E. and H. Selye. *Endocrinology* **54**: 550. 1954.
7. Kennedy, G. C. *British Medical Journal* **13**: 67. 1957.
8. Masson, G. M. C., S. B. Koritz and F. G. Peron. *Endocrinology* **62**: 229. 1958.

PHASE RELATIONS OF 24-HOUR PERIODICITIES IN BLOOD CORTICOSTERONE, MITOSES IN CORTICAL ADRENAL PARENCHYMA, AND TOTAL BODY ACTIVITY[1,2]

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ABSTRACT

Under conditions standardized for analysis of 24-hour periodicity, with light from 06:00 to 18:00 alternating with 12 hours of darkness, corticosterone in mouse serum undergoes reproducible 24-hour changes, with peak blood levels at about 16:00. Significant differences in phase characterize the 24-hour rhythms in serum corticosterone and in mitoses of cortical adrenal parenchyma; under the same lighting conditions, the peak of the latter occurs at about midnight. The corticosterone rhythm leads in phase the rhythm in gross motor activity and lags behind the eosinophil rhythm, in the absence of intentional stimulation other than daily routine. As compared to the significant change in blood corticosterone level along a 24-hour scale, the immediate effect of audiogenic stimulation upon serum corticosterone is slight. It consists of a small rise following stimulation at the time of the daily "corticosterone low" and is negligible after stimulation at the time of "corticosterone high." The daily rhythm in blood level of adrenal 11-oxycorticoid in nocturnal mice, as compared to predominantly diurnal man is comparable in amplitude but differs in its environmental phase relations, indicating a species difference in mode of synchronization of rhythm.

DAILY changes in corticosterone content of mouse serum were studied by adapting several chemical methods (1–3) for use on small samples. The occurrence and the timing of daily changes in blood corticoid of mice had earlier been suggested from eosinophil behavior under various standardized conditions (4–9) and blood-corticoid periodicity had been interpreted as underlying a 24-hour adrenal cycle, traced to the gland itself by

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spot-checks of mitotic counts in its parenchyma (10, 11). Herein, the periodicity and phase of adrenal mitoses in relation to rhythms in blood corticosterone and eosinophils will be described on the basis of more frequent sampling along a 24-hour scale, as a possible time base for future studies of hormone production and release. Since corticoids in the urine or blood of man or monkey exhibit 24-hour variations (12–15), corresponding changes in nocturnal rodents are also of interest to comparative endocrinology. In man, morning eosinopenia (16), as well as a rise in blood corticoids (17), is initiated before the habitual time of awakening. The phase relations of the rhythms in blood corticosterone, on one hand and in gross body activity on the other, in mice studied under standardized circumstances thus deserve interest. Will the daily rise in blood corticosterone of mice lead the onset of gross body activity, as an endocrine entity may do (7, 8), or will it lag behind as a mere "reaction to activity?" The suggestion that an adrenal cycle is preparatory for daily activity and that its period does not depend directly or solely upon environmental control (5–8, 18) differs from unqualified references 1) to daily changes in corticoid levels as "responses to the stresses of daily life" and 2) to a "basal" level of adrenal cortical activity as a characteristic of rest and/or sleep.

The role of the adrenal cycle, in functional integration and environmental adaptation has been discussed (7, 8, 18); this endocrine entity persists under conditions which obliterate the estrus cycle of mice (5) and it develops earlier than the menstrual cycle in human beings (19). The main point of this paper is a description of the more detailed phase relations of some parameters of the adrenal cycle in mice, a documentation of mechanisms remaining beyond our scope.

MATERIALS AND METHODS

Inbred mice of the C(Bagg albino) and the D_8 (Dilute Brown, Subline 8) stocks were used, sex and age being given with the results. Blood eosinophil counts were done with assembly-line procedures (21), on separate groups of mice, killed at 4-hour intervals during a 24-hour period. From the same mice, after cervical dislocation, the adrenals were removed and mitoses in cortical parenchyma were counted in histologic sections, 5 μ thick, stained with Heidenhain's iron hematoxylin and faintly counterstained with eosin. The mitotic counts were done under oil immersion, in nearly equatorial sections of the cortex (at least 10 μ apart from each other). Blood corticosterone was determined on $\sim$0.5 cc. of serum, pooled from groups of 5–15 mice. Two methods were used, a double isotope derivative assay (2), briefly P, and a fluorometric technique (3), briefly S; method S is known to lack the specificity of P, which is more rigorous but more time consuming than S. The much higher absolute values for corticosterone obtained (even on true duplicates) with S cannot be interpreted as such and will be discussed only after conversion into "relatives" (% of 24-hour mean) (cf. Fig. 1).

Corticosterone would appear to be the major steroid product secreted by the mouse adrenal cortex.[4] Bush (22) had earlier reported that in the rodent, the principal adreno-

[4] For a detailed analysis of steroids in mice bearing an ACTH producing tumor see reference 31.

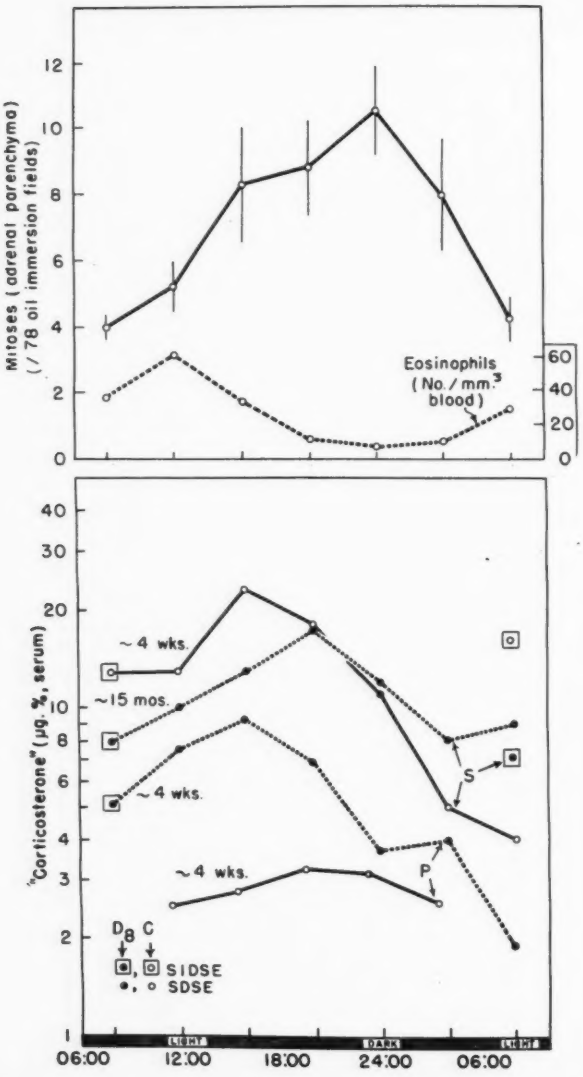

FIG. 1. Some parameters of the daily adrenal cycle in the mouse. Absolute values. Top: Mitoses of adrenal cortex and blood eosinophils, in male C mice, 4 weeks of age. Bottom: 24-hour changes of corticosterone, determined by different methods, P and S (see text and Figure 2). Each point based upon separate groups of mice (serially independent sampling as to animals). SIDSE = serially independent sampling as to environment (groups of mice from experimental rooms not entered during the 24 hours prior to sampling); SDSE = serially dependent sampling as to environment (for a discussion of such sampling procedures see reference 18).

cortical steroid is corticosterone; he could not detect any hydrocortisone. One of the present authors (Ralph E. Peterson) has assayed pooled mouse serum by the isotope derivative and an isotope derivative dilution method and found 7 μg.% corticosterone, but no hydrocortisone. In addition, isotope derivative and isotope derivative dilution assays have been performed on mouse adrenal vein blood. (Dr. William Tullner kindly carried out the adrenal vein cannulations in the mice.) Hydrocortisone could not be detected. Corticosterone levels ranged from 500 to 700 μg.%, and aldosterone levels were 10 to 20 μg.%.

Body activity was recorded in ten electronic actometers, consisting of cages mounted on vertically compliant suspensions, resonant at 90 hertz, which actuated motors from electro-dynamic speakers to provide velocity-sensitive transducers (23). Electrical outputs were amplified and after amplitude discrimination actuated digital registers, which were simultaneously photographed at hourly intervals.

All of the experiments reported herein were carried out on singly-housed animals with Purina dog chow and tap water available *ad libitum* from weaning and throughout the investigation. For nine days prior to each study, and throughout the investigations, the mice were kept at 24.5 ± .5° C, in light from 06:00 to 18:00, alternating with twelve hours of darkness.

RESULTS AND DISCUSSION

Figure 1 shows the absolute values obtained for mitoses in cortical adrenal parenchyma, for blood eosinophils and for serum corticosterone. On the curves for corticosterone, each point represents one determination on a serum pool from a group of animals, killed at a given time of day. By contrast, the curves of eosinophils, mitoses, and body activity consist of points representing a mean value, computed from the individual data. As group phenomena, the 24-hour rhythms studied stand out clearly in Figure 1; only the curve for corticosterone at the bottom of this figure seems flat, but this curve is incomplete, since the samples at 08:00 were inadvertently lost; the lack of data at the usual time of minimum value may have contributed to the lesser amplitude of that curve.

These studies were carried out under standardized conditions, but earlier reference to the absence of intentional stimulation other than daily routine deserves comment. It is true that separate subgroups of comparable animals were studied at different time points. But mice used later in the day were unintentionally disturbed by 1) noise which could not be avoided while removing the mice studied earlier from their cages, and by 2) the disruption of the dark period by the use of a shaded flashlight (for locating the animals). Moreover, visual and other stimulation (e.g., that associated with the presence of the experimentor in the mouse room) also can affect the experimental animal (24, 25). Thus, by using different mice at different time points, only serial independence as to animals was achieved while the effect of serial dependence as to experimental room remains to complicate the data. As a spot-check on the later effect (serial dependence as to environment), in some of the series on corticosterone, at the last time point (08:00), comparable animals housed in a different but comparable room also were studied and such data are included in Figure 1. At 08:00, the end of study, corticosterone values were usually, but not invariably, lower in mice disturbed by prior sampling at 4-hour intervals than in animals kept undisturbed in a different room. The effect of unintentional stimulation of mice by the experimentors was not analyzed statistically in the limited number of pertinent corticosterone pools, but it was highly significant in comparable data on several other rhythms (e.g., mitoses in ear epidermis).

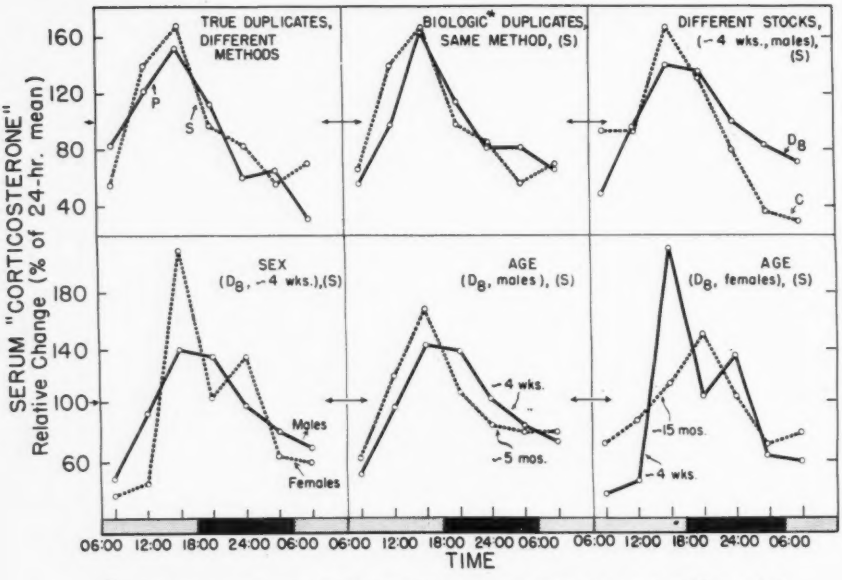

FIG. 2. Daily change in serum corticosterone of mice. "Relative" values (% of 24-hour mean).

Figure 2 includes additional data on corticosterone in mouse serum, all expressed as per cent of 24-hour mean. Reasonable agreement between the daily changes depicted by methods P and S may first be seen from determinations on true duplicate samples. Even though, in absolute terms, higher values are obtained by S than by P, almost certainly due to the lesser specificity of S, the curves yielded by the two methods become comparable after conversion of data to per cent of series mean. "Biologic" duplicates, i.e., two sets of determinations made by method S on two subgroups of comparable mice, killed at 4-hour intervals during the same 24-hour period are also in good agreement. Figure 2 shows, further, the relative curves of "corticosterone" for animals of different sex, but of the same stock and age, as well as stock and age comparisons (some curves are reproduced in several comparisons). If, as seems likely, "relative" results obtained by S depict changes in corticosterone level, it can be suggested first, that in mice of two stocks, of both sexes and of widely different ages, 24-hour changes characterize the level of corticosterone in blood; and second, that the daily peak in serum corticosterone of mice occurs at about 16:00, under conditions of light from 06:00 to 18:00 alternating with twelve hours of darkness (for the data on D_8 females, ~15 months of age, see discussion below).[5]

[5] The two groups of mice were different in age, sex, stock, and in that the D_8 mice had the Bittner mammary tumor agent, while the C mice did not have the agent.

As compared to the 24-hour periodic changes in serum corticosterone, occurring in mice on a standardized daily routine, the extent of change following exposure to audiogenic stimulation was small. Noise-sensitive D_8 mice, about five weeks of age, were studied in a specially constructed stimulator;[6] at two phases of daily cycle, they were exposed for 60 seconds to noise of about 117 db (above 0.0002 dynes/sq.cm. r.m.s. pressure), yielded by a noise generator. Individual animals were transferred from their cages to the stimulator within less than 30 seconds. Upon completion of stimulation, the animals were killed and pools of serum collected for corticosterone determination. Serum from animals with and without audiogenic convulsions was pooled separately, as was serum from comparable animals not subjected to audiogenic stimulation. Determinations were done with P and S, on separate sets consisting of the three types of pools compared; the results of the two methods expressed as % change from the nonstimulated value were roughly in agreement.

Stimulation in the morning, not followed by convulsion, had a slight effect: an average increase in serum corticosterone of 19.6 ± 6.9 per cent was found for three pools obtained after stimulation, as compared to three paired pools from nonstimulated mice, when the latter values were equated to 100 per cent. By contrast, five similar comparisons of values after stimulation in the evening, not followed by convulsion, with corresponding values on nonstimulated mice revealed no effect: two pools after stimulation were higher, two were lower, and one was equal to a paired pool from nonstimulated animals. With the value for nonstimulated mice equated to 100 per cent, the corresponding difference of -2.8 ± 16 seems negligible. Furthermore, following stimulation in the evening, three pools also were collected from animals exhibiting audiogenic seizures. The mean difference between the corticosterone level in these pools, on one hand, and that in paired pools from nonstimulated animals was negligible, i.e., -4.8 ± 13 per cent.

Figure 3 shows 24-hour changes in gross body activity of two different populations of mice kept in different but comparable rooms; the sensitivity of the actometers was adjusted to record "washing" movements as well as locomotion. As was anticipated (26–28), both curves exhibit significant periodicity, but the peaks occur at different times. Each curve is based upon data from five mice, in individual actometers, kept in a room housing also comparable animals used for the collection of serum for corticoid determinations. Each activity value represents an integration of actometer counts over a given 4-hour period and is plotted at the midpoint of that period. The corticosterone values describe discrete 4-hourly samples; peaks or troughs lying between the times chosen for sampling thus may have been obscured. This limitation notwithstanding, it is of interest to counterpose

[6] To be described elsewhere, constructed by K. Shimada with advice from Dr. E. Hartig and R. P. Featherstone.

to the curve in body activity, the corresponding curve for blood "corticosterone" (S). Such a comparison in Figure 3 suggests that a) the curve for "corticosterone" rises before that for body activity, b) the peak for "corticosterone" precedes that for body activity, and that c) "corticosterone" levels have dropped below their day mean at a time when body activity is still above this mean (100 per cent line, Fig. 3). Along a 24-hour scale, the changes in serum corticosterone thus appear to lead in phase those in gross body activity and almost certainly do not lag.

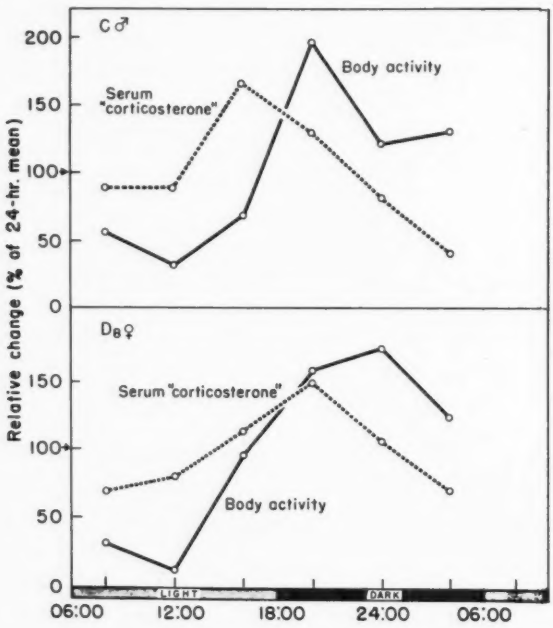

FIG. 3. Daily changes in gross body activity of two populations of mice[5] in relation to corresponding changes in serum corticosterone. The latter rhythm leads the former.

Reciprocal phase relations among 24-hour rhythms in blood eosinophils, serum corticosterone, and mitoses of adrenal cortical parenchyma may be examined in Figure 4. The top half of the figure shows that in the mouse a rise in eosinophils occurs before that in corticosterone, daily eosinopenia, in turn, lagging behind the start of corticosterone rise. Accordingly, the blood eosinophil peak occurs before that in corticosterone, while the peak of mitoses in adrenal cortex occurs later. The present, more complete curve for cortical mitoses agrees well with earlier results of spotchecks (10, 11). The relation of mitosis to secretion in the gland has previously been discussed on the basis of time relations between rhythms in eosinophils and cortical mitoses (10, 11); those earlier inferences seem to be supported by

the phase relation of the curves for cortical mitoses on one hand and for serum corticosterone on the other. It is of further interest that the mitotic rhythm of the adrenal cortex is substantially out of phase with rhythms of mitoses in liver parenchyma or skin epidermis (29); the peak of the latter rhythms occurs during the middle of the light period, in animals of comparable stock and age, i.e., at a time when the adrenal cortical rhythm is below its day mean.

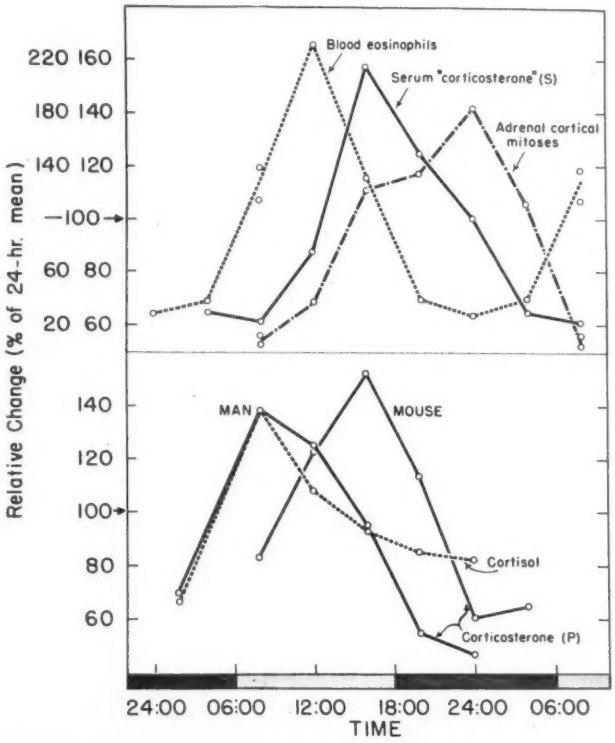

FIG. 4. Some parameters of the daily adrenal cycle. "Relative" values (% of 24-hour mean). The lighting regimen on abscissa applies strictly only to data on the mouse.

Lastly, a species difference in corticosterone rhythm between human beings on one hand, and mice on the other, is shown in the lower half of Figure 4. Data obtained by one of us on daily changes in blood cortisol and corticosterone of human beings (30) are graphed against the changes reported herein for serum corticosterone in the mouse. The curves of corticosterone in nocturnally active rodents and predominantly diurnal man are out of phase, thus revealing a difference in mode of synchronization with the environment.

REFERENCES

1. Peterson, R. E.: *J. Biol. Chem.* **225:** 25. 1957.
2. Kliman, B. and R. E. Peterson: *Fed. Proc.* **17:** 255. 1958.
3. Silber, R. H., R. D. Bush, and R. Oslapas: *Clin. Chem.* (In press).
4. Halberg, F. and M. B. Visscher: *Proc. Soc. Exp. Biol. and Med.* **75:** 846. 1950.
5. Halberg, F. and M. B. Visscher: *Endocrinology* **51:** 329. 1952.
6. Halberg, F., M. B. Visscher and J. J. Bittner: *Am. J. Physiol.* **174:** 313. 1953.
7. Halberg, F. *Journal Lancet* **73:** 20. 1953.
8. Halberg, F.: *Acta Medica Scandinavica, Suppl.* **307:** 117. 1955.
9. Brown, H. E. and T. F. Dogherty: *Endocrinology* **58:** 365. 1956.
10. Mühlemann, H. R., T. M. Marthaler and P. Loustalot: *Proc. Soc. Exp. Biol. and Med.* **90:** 467. 1955.
11. Halberg, F., M. J. Frantz and J. J. Bittner: *Anat. Rec.* **129:** 349. 1957.
12. Pincus, G.: *J. Clin. Endocrinology* **3:** 195. 1943.
13. Appel, W. and K. J. Hansen: *Dtsch. Arch. f. klin. Med.* **199:** 530. 1952.
14. Doe, R. P., E. B. Flink and M. G. Flint: *J. Clin. Endocrin. and Metab.* **14:** 774. 1954.
15. Migeon, C. J., A. B. French, L. T. Samuels and J. Z. Bowers: *Am. J. Physiol.* **182:** 462. 1955.
16. Halberg, F., R. Engel, E. A. Treloar and R. J. Gully: *A.M.A. Arch. Neurol. and Psych.* **69:** 462. 1953.
17. Tyler, F. M., C. Migeon, A. A. Florentin and L. T. Samuels: *J. Clin. Endocrinology and Metab.* **14:** 774. 1954.
18. Halberg, F.: *Z. f. Vitamin-, Hormon- und Fermentforschung* (In press).
19. Halberg, F. and R. Ulstrom: *Proc. Soc. Exp. Biol. and Med.* **80:** 747. 1952.
20. Halberg, F. and A. N. Stephens: *Fed. Proc.* **17:** 439. 1958.
21. Halberg, F., J. J. Bittner and M. B. Visscher: *Blood* **6:** 832. 1951.
22. Bush, J. E.: *J. Endocrinology* **9:** 95. 1953.
23. Bruss, R. T., E. Jacobsen, F. Halberg, R. A. Zander and J. J. Bittner: *Fed. Proc.* **17:** 21. 1958.
24. Gangloff, H. and M. Monnier: *EEG Clin. Neurophysiol.* **8:** 623. 1956.
25. Halberg, F., H. R. Zander, M. W., Houglum and H. R. Mühlemann: *Am. J. Physiol.* **177:** 361. 1954.
26. Szymanski, J. S.: *Arch. ges. Physiol.* **158:** 343. 1914.
27. Johnson, M. S.: *J. Exp. Zool.* **82:** 315. 1939.
28. Park, O.: *Ecological Monographs* **10:** 485. 1940.
29. Halberg, F., C. P. Barnum, R. H. Silber and J. J. Bittner: *Proc. Soc. Exp. Biol. and Med.* **97:** 897. 1958.
30. Peterson, R. E.: *J. Clin. Endocrin. and Metab.* **17:** 1150. 1957.
31. Wilson, H., J. J. Borris and R. C. Bahn: *Endocrinology* **62:** 135. 1958.

THE INFLUENCE OF CORTISONE GLYCINATE AND TESTOSTERONE GLYCINATE ON ADRENALECTOMIZED GOLDEN HAMSTERS AND CONTROL HAMSTERS

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ABSTRACT

The biological properties of cortisone glycinate and testosterone glycinate—two highly water soluble synthetic steroids—and of cortisone were compared in adrenalectomized and non adrenalectomized golden hamsters (Mesocricetus auratus). Special attention was paid to their effect on body and liver weight and liver components. Testosterone glycinate and also large doses of cortisone—1000 μg./100 gm. body weight—were found to have a toxic effect on golden hamsters whether adrenalectomized or not, while the toxicity of cortisone glycinate was very low. In contrast to testosterone glycinate cortisone and especially cortisone glycinate were effective in sustaining life in adrenalectomized animals. Cortisone caused an increase, testosterone glycinate a decrease in liver weight both in adrenalectomized and non adrenalectomized hamsters. With cortisone glycinate a normal development took place. The glycogenic properties of cortisone and of cortisone glycinate—but only after prolonged administration—were apparent both in adrenalectomized and in control hamsters, while testosterone glycinate did not posses any activity in this respect. The fat concentration of the liver was increased following administration of cortisone, but hardly responded to administration of both the other compounds. Cortisone and testosterone glycinate treatment of adrenalectomized animals caused a decrease in phospholipid concentration in the liver, whereas cortisone glycinate tended to maintain normal levels. Suggestions are presented in an attempt to explain these different effects.

THE biological properties of certain aminosteroids have been discussed in previous publications (1, 2, 3a, 3b, 4). Steroids linked with amino-acids were produced chiefly in order to increase the solubility of steroids. It was believed that—within the organism—these N-steroids would be rapidly hydrolysed to liberate the corresponding steroid. Subsequent experiments with such compounds showed that the biological action of the aminosteroids was not entirely identical with that of free steroids. Testosterone glycinate has no androgenic properties as compared with free testosterone; no testosterone glycinate was found unchanged in the urine (5). Even certain steroids linked with succinic acid (e.g. cortisone hemisuccinate) have biological properties different from those of free steroids (6). Jacoby and Tompkins (7) demonstrated, that hemisuccinates could be reduced without previous saponification.

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[1] Read in part before a meeting of the Congress of Swiss and German Societies for Physiological Chemistry, Basel, September 1957.

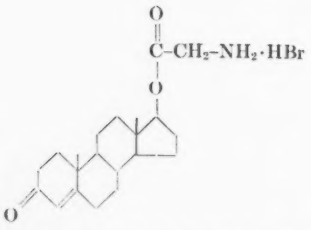
This paper presents the results of an investigation into the influence of two synthetic steroids—cortisone glycinate and testosterone glycinate (8)—on body weight, liver weight and liver composition in adrenalectomized golden hamsters (Mesocricetus auratus) and non-adrenalectomized controls.

METHODS

Experimental animals used where golden hamsters, as these are known to lack accessory adrenals (9, 10). The animals were separated according to sex and then divided into adrenalectomized groups and control groups, each group including at least 20 animals. The approximate body weight was 40–60 gm., and the approximate age 8–10 weeks. The animals were given a standard diet ad libitum and received daily injections of the N-steroid tested (200 μg./100 gm. body weight) and of cortisone (1000 μg./100 gm. body weight). Experiments were carried out over a period of 44 days; one group of adrenalectomized animals and one group of controls were sacrified for correlative analysis on the 7th, 14th, 24th, 34th and 44th day. In animals given steroids with a toxic effect or lacking life-sustaining properties, experiments naturally had to be terminated after 14 days or in same cases after 7 days.

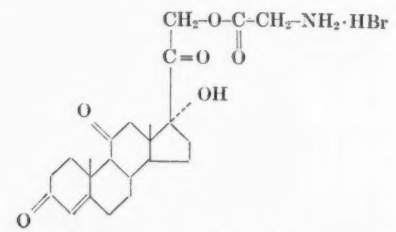
Plan of investigation

Experimental animals: golden hamsters (Mesocricetus auratus)
Divided into: adrenalectomized animals and controls
Treated with: cortisone (1000 μg./100 gm. body weight daily)
cortisone glycinate (200 μg./100 gm./day)
testosterone glycinate (200 μg./100 gm./day)
Interval until death: 7, 14, 24, 34, 44 days (animals sacrified by decapitation)
Factors determined:
a) body
1. initial and ultimate body weight
2. ratio between body weight and liver weight
b) liver
1. liver weight
2. nitrogen
3. phosphorus
4. glycogen
5. fat
6. phospholipids
7. cholesterol
8. bile esters



Testosterone glycinate hydrobromide

UVλ max alcohol	241 mμ.
Molecular extinction alc.	14.700
UVλ max water	248 mμ.
Molecular extinction water	14.100
Water solubility 200 mg./ml.	



Cortisone glycinate hydrobromide

UVλ max alcohol	238 mμ.
Molecular extinction (alc.)	14.700
UVλ max water	245 mμ.
Molecular extinction water	14.500
Water solubility 60 mg./ml.	

In alcohol, testosterone glycinate hydrobromide and cortisone glycinate hydrobromide have a UV absorption maximum of about 240 mμ., which is characteristic of Δ^4,3-ketosteroids. Both are highly water soluble, in which respect they have a great advantage over cortisone or other steroids.

RESULTS

Normal untreated golden hamsters showed an increase in body weight up to 16% after 34 days; after adrenalectomy, animals showed a decrease in body weight of about 26%, and died after 5–7 days.

Testosterone glycinate was found to have a toxic effect on golden hamsters whether adrenalectomized or not. The majority died after 7 days, showing a slight decrease in body weight (about 8%). Testosterone glycinate proved to have no life-sustaining properties in the adrenalectomized animals, which died within 5–7 days after administration showing a decrease in body weight comparable to that in the control group.

Large doses of cortisone (1000 μg./100 gm. body weight) also had a toxic effect. The loss of weight accompanying administration of this substance has previously been reported by other authors. Prolonged administration of cortisone was found to be fatal. Most of the animals died after 20–30 days' treatment, showing a decrease in body weight of about 30%. Cortisone sustained life in adrenalectomized animals up to 34 days but failed to stop the loss of weight.

Cortisone glycinate did not affect normal development of body weight in golden hamsters during the first 24 days of administration. The increase in body weight throughout this period roughly corresponded with that in the controls. After prolonged treatment (exceeding four weeks) there was a very marked increase in body weight, up to 40%. This phenomenon was associated with an increase in liver glycogen concentration and premature maturation of reproductive organs. Cortisone glycinate was very effective in sustaining life in adrenalectomized animals, although there was a decrease in body weight during the first two postoperative weeks. The animals regained initial weight values and exceeded them, however, 4–5 weeks after adrenalectomy (Fig. 1).

The influence of these synthetic steroids on the liver was investigated with special reference to their effect on glycogen, fat phospholipid, cholesterol, cholesterol-ester and phosphorus concentrations.

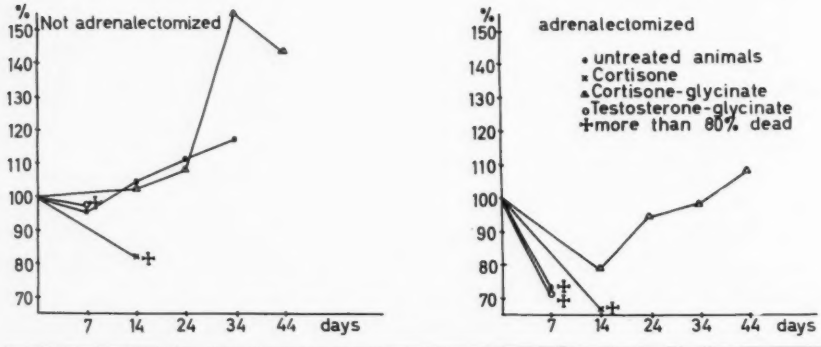
Findings showed that the average liver weight in normal golden hamsters was 3 gm. This was naturally reduced after adrenalectomy, chiefly due to loss of water and organic components. Liver weight was also reduced following administration of testosterone glycinate, both in adrenalectomized animals and in controls (Fig. 2).

Cortisone caused an increase in liver weight in adrenalectomized animals and controls. As a result of the decrease in body weight following administration of cortisone the percentual increase in liver weight relative to body weight was even more marked.

Cortisone glycinate slightly increased liver weight but only after prolonged administration (exceeding two weeks). Adrenalectomized animals treated with cortisone glycinate showed a decrease in liver weight within

the first two weeks but regained initial weight values when administration was prolonged.

The ratio between body weight and liver weight is a useful aid in distinguishing between loss of body weight and loss of liver weight. In normal golden hamsters the ratio was found to be 6.4; a reduction of this ratio following adrenalectomy (down to 4.5) shows that the loss of liver weight exceeds the loss of body weight. It should be pointed out that all figures were statistically computed; the statistical significance of the deviation was also determined.



Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ
Not adr. ect.	7	20	95,3 ± 10,7			20	96,2 ± 15,4		
"	14	20	104,5 ± 12,7	10	102,5 ± 9,42			10	82,1 ± 25,4
"	24	20	111,1 ± 11,6	10	107,7 ± 10,8				
"	34	20	116,7 ± 10,0	10	155,2 ± 24,4				
"	44			10	143,5 ± 22,7				
adr. ect.	7	30	73,3 ± 15,66			40	71,8 ± 13,3		
"	14			10	78,9 ± 17,2			10	67,1 ± 16,9
"	24			10	94,6 ± 28,0				
"	34			20	98,1 ± 31,9				
"	44			20	108,1 ± 27,5				

FIG. 1. Body weight curve in adrenalectomized golden hamsters and in controls. Values represent percentage increase or decrease relative to initial weights.

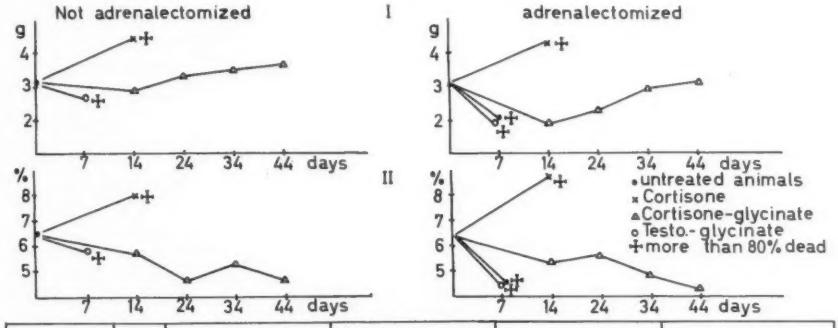
Liver glycogen concentration: this was about 13 mg. glycogen per gramme fresh liver weight in untreated controls—a figure corresponding with that found in other laboratory animals. The fall in glycogen concentration following adrenalectomy is attributable to glycogenolysis as a result of the operation, and to the lack of adrenal hormones which prevents the liver from forming new glycogen. Testosterone-glycinate had no glycogenic effect either in adrenalectomized or in control animals.

The glycogenic properties of cortisone glycinate were apparent both in adrenalectomized and in control hamsters but only after prolonged administration (exceeding 24 days). Cortisone had marked glycogenic prop-

erties event within a week of its administration and even in adrenalectomized animals (Fig. 3).

Overbeek *et al.* (11) investigated glycogen activity in starving rats and, under special conditions, established a limited glycogenic effect of cortisone glycinate. Cortisone hemisuccinate resembled glycinate in this respect.

The fat concentration in the liver in normal golden hamsters amounted to 30 mg./gm. liver weight. This concentration was increased following cortisone administration, and slightly decreased following administration of testosterone glycinate or cortisone glycinate.

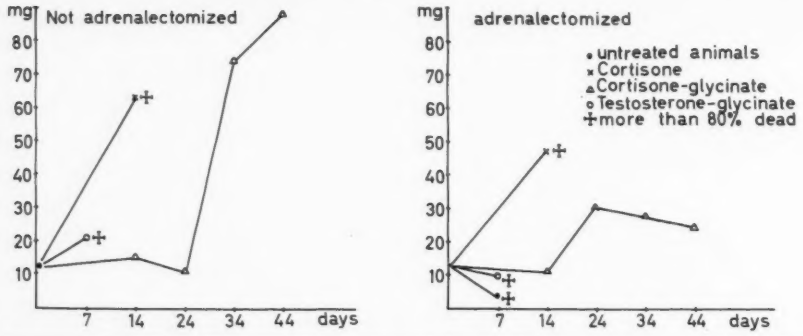


Treatment	Days	Controls		Cort.-glycinate		Testo-glycinate		Cortisone	
		Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ
Not adr.ect.	7	28	3,13 ± 0,55			20	2,691 ± 0,64		
"	14			10	2,923 ± 1,41				4,456 ± 1,24
"	24			10	3,368 ± 0,99				
"	34			10	3,512 ± 1,31				
"	44			10	3,636 ± 1,07				
adr. ect.	7	30	2,096 ± 0,55			40	2,030 ± 0,57		
"	14			10	1,894 ± 0,92				4,337 ± 1,44
"	24			10	2,286 ± 0,98				
"	34			20	2,933 ± 1,42				
"	44			20	3,137 ± 1,40				

FIG. 2. Liver weight curve in adrenalectomized hamsters and in controls. The slope of the absolute values which are given in the table is shown in part I. Part II shows the values expressed by the ratio between liver weight and body weight.

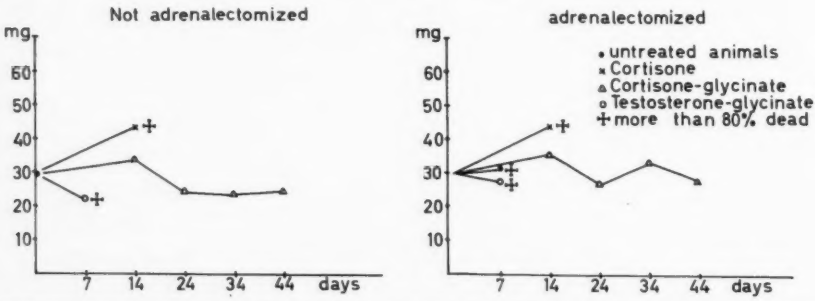
The liver fat concentration following adrenalectomy was 30 mg./gm. fresh liver weight—identical with the concentration prior to adrenalectomy in view of the decrease in liver weight which follows adrenalectomy, however, the total fat concentration of the liver was evidently reduced after the operation.

The fat concentration of the liver was increased following administration of cortisone, even in adrenalectomized animals, but it hardly responded to administration of testosterone glycinate or cortisone glycinate (Fig. 4). Kendall and Heilman (12) demonstrated that administration of several other steroids to mice was followed by a decrease in body weight



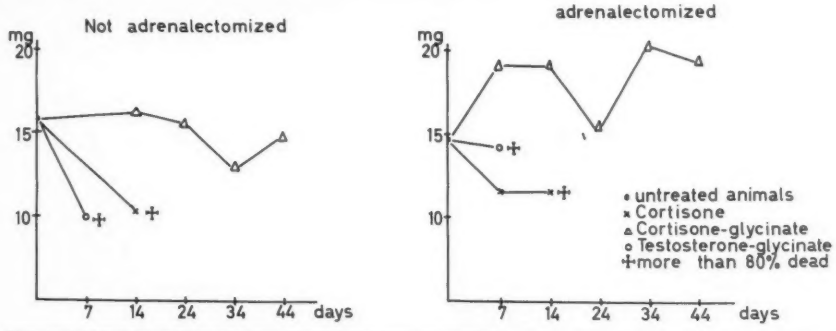
Treatment	Days	Controls		Cort.-glycinate		Testo-glycinate		Cortisone	
		Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ
Not adr. ect.	7	28	12,7 ± 4,36			20	21,0 ± 8,5		
"	14			10	15,1 ± 7,5			10	63,0 ± 42,8
"	24			10	11,0 ± 1,5				
"	34			10	74,0 ± 4,3				
"	44			10	81,3 ± 32,6				
adr. ect.	7	30	4,15 ± 4,04			40	10,1 ± 5,68		
"	14			10	11,4 ± 9,5			10	47,3 ± 40,8
"	24			10	31,7 ± 13,0				
"	34			20	27,5 ± 14,5				
"	44			20	24,9 ± 17,0				

FIG. 3. Liver glycogen curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.



Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ
Not adr.ect.	7	28	29,6 ± 7,04			20	22,0 ± 10,3		
"	14			10	34,1 ± 15,74			10	43,37 ± 14,4
"	24			10	24,5 ± 7,21				
"	34			10	23,4 ± 8,0				
"	44			10	23,9 ± 10,5				
adr.ect.	7	30	31,6 ± 7,29			40	27,9 ± 6,18		
"	14			10	36,2 ± 1,98			10	44,56 ± 12,4
"	24			10	27,1 ± 10,68				
"	34			20	33,0 ± 13,4				
"	44			20	27,6 ± 13,1				

FIG. 4. Liver fat curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.



Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ
Not adr. ect.	7	28	15,8 ± 2,21			20	10,0 ± 4,59		
"	14			10	16,22 ± 9,41			10	10,44 ± 4,54
"	24			10	15,60 ± 5,48				
"	34			10	12,80 ± 2,64				
"	44			10	14,80 ± 5,8				
adr. ect.	7	30	14,8 ± 2,82	10	19,3 ± 10,05	40	14,3 ± 4,44	10	11,7 ± 5,37
"	14			10	19,2 ± 14,24			10	11,7 ± 2,85
"	24			10	15,2 ± 8,12				
"	34			20	20,5 ± 12,38				
"	44			20	19,5 ± 11,04				

FIG. 5. Liver phospholipid curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.

and an increase in body fat (from 3 to 18%). Fatty degeneration of the liver has likewise been reported.

The phospholipid concentration of the liver in the animals under investigation amounted to about 50% of the fat concentration, the remaining 50% consisting of about 1/30 cholesterol, 1/50 cholesterol-ester and further neutral fat and fatty acids.

Administration of cortisone to adrenalectomized animals caused a decrease in phospholipid concentration per gramme liver weight. This was also the case with testosterone glycinate, whereas cortisone glycinate tended to maintain or to increase the phospholipid concentration (Fig. 5).

An increase in cholesterol concentration per gramme liver weight was seen following adrenalectomy in the control group, whereas a decrease occurred following administration of testosterone glycinate and cortisone glycinate. The phosphorus level of the liver was determined on the basis of inorganic phosphorus. There was an increase during glycogenolytic processes (e.g. adrenalectomy), and only a slight increase during glycogenic processes (cortisone administration).

DISCUSSION

In the past few years there has been an increased interest in steroid-protein complexes (13–19) and in the steroid-amino-acid compounds (3a, 3b) as well as in the influence of some known steroids upon the liver

composition (20–31). In an investigation of the question regarding the properties of such substances the use of synthetic crystalline compounds may be valuable.

Testosterone glycinate was found to have a toxic effect, whereas the toxicity of cortisone glycinate was very low in golden hamsters. Comparative investigations into larger doses of cortisone showed that cortisone is less toxic than testosterone glycinate but more toxic than cortisone glycinate. Cortisone glycinate was as effective as cortisone in life-sustaining experiments in adrenalectomized animals. Testosterone glycinate showed a complete lack of life-sustaining properties.

Both cortisone and testosterone glycinate caused a decrease in body weight, most marked in the case of cortisone although, as pointed out, testosterone glycinate was more toxic. Cortisone glycinate caused no decrease in body weight, normal development continuing until the 24th day. Weight development was accelerated after the 24th day, an increase to 40% being determined within 10 days. An accelerated increase in liver glycogen was likewise observed. It is difficult to explain this phenomenon. The golden hamster perhaps does not possess a specific enzyme to hydrolyse cortisone glycinate into free cortisone and glycine. Such an enzyme may be formed, however, after prolonged administration of cortisone glycinate. This theory would seem to be supported by the literature, which includes some reports on similar cases of adaptive enzyme formation. The change in the liver glycogen concentration following prolonged administration of cortisone glycinate shows a distribution curve identical with that of free cortisone within the first week of administration. Cortisone glycinate showed eosinophile depressing properties, as also in the case of cortisone. The problem becomes even more complicated when the body weight and fat composition of the liver are taken into account. Cortisone administration causes a decrease in body weight, whereas cortisone glycinate increases body weight. Only minor fluctuations in the liver fat concentration were seen following treatment of animals with testosterone glycinate or cortisone glycinate, whereas cortisone caused a rise in fat concentration, prolonged administration of large doses giving rise to fatty infiltration of the liver. The phospholipid content was decreased after cortisone or testosterone glycinate treatment, remaining within normal limits after cortisone glycinate administration. Amino-acid esters of steroids therefore would seem to have special biological properties in some respects. Certain other cortisone derivatives—e.g. cortisone hemisuccinate—showed antigenic properties when combined with bovine serum albumin in rabbits (32). Some testosterone derivatives with substitution in C_3 or C_{17} antagonize testosterone, e.g. testosterone-17-chlorocarbonate (33) and testosterone-3-(O-carboxylmethyl)-oxime (32). This corroborates our observation that cortisone glycinate showed a gonadotropic effect on golden hamsters, and it may be explained on the basis of a secondary effect. Schneider and Frahm (34) also described pituitary stimulation effected by some aminosteroids isolated from urine.

The biological properties of cortisone glycinate and some nitrogen-bearing steroids isolated from aqueous adrenal extracts require further investigation with a view to possibilities of practical application. Such compounds probably interfere with the fat metabolism, as suggested by Schmitz and Hilgetag (35) and Schmitz and Kühnau (36) with reference to certain adrenal fractions. The low toxicity and the biological properties in general, furthermore, render cortisone glycinate a suitable new synthetic compound for introduction into the series of applied hormonal preparations.

REFERENCES

1. Voigt, K. D.: Habilitation-schrift, 1955, Hamburg.
2. Voigt, K. D. and W. Schroeder: *Acta endocrinol.* **21:** 343. 1956.
3a. Voigt, K. D. and G. Kallistratos: Symposion 5. dtsch. Ges. f. Endocrinologie, Freiburg. 1957.
3b. Voigt, K. D. and G. Kallistratos: *Endokrinologie* **35:** 56. 1957.
4. Schroeder, W. and K. D. Voigt: *Acta endocrinol.* **27:** 110. 1958.
5. Overbeek, G. A.: Personal communication.
6. Kracht, J.: Personal communication.
7. Jacoby, W. B. and G. M. Tomkins: *Science* **123:** 940. 1956.
8. van Dorp, D. A., H. P. de Jongh and Boldt: 1st synthesis. 1957.
9. Küchmeister, H. and G. Assmann: *Klin. Wschr.* 1043. 1952.
10. Schroeder, W. and K. Bohle: *Acta endocrinol.* **24:** 8. 1957.
11. Overbeek, G. A., J. de Visser and J. van der Vies: International Congress on Therapy, Utrecht, June 1957.
12. Kendall, E. C. and F. R. Heilmann: *Proc. staff. meet. Mayo clinic* **31:** 454. 1956.
13. Szego, C. M.: *Colloquia on Endocrinology, Ciba Foundation* **11:** 286. 1957.
14. Areshkina, L. J., V. N. Bukin, N. N. Erofeteva and E. P. Skorobogatoba: *Biochimija* (*USSR*) **22:** 384. 1957.
15. Bukin, V. N. and L. J. Areshkina: *Biochimija* (*USSR*) **15:** 448. 1950.
16. Deborin, G. A. and L. B. Gorbatscheva: *Biochimija* **18:** 618. 1953.
17. Troitskii, G. V. and L. S. Tarasova: *Biochimija* **20:** 19. 1955.
18. Troitskii, G. V. and D. A. Sorkina: *Biochimija* **21:** 784. 1956.
19. Vendt, V. P.: *Vitamini* (*USSR*) **2:** 25. 1956.
20. Aterman, K. and N. D. Ahmad: *Lancet* **1:** 71. 1953.
21. Benton, D. A., A. E. Harper and C. A. Elvehjem: *J. Biol. Chem.* **218:** 693. 1956.
22. Bernard, K. and F. Lindlar: *Helv. physiol. pharm. Acta* **14:** 113. 1957.
23. Blaupin, O. and A. Aschkenazy: *Comtes rend. soc. biol.* **149:** 1886. 1955.
24. Dulin, W. E.: *Proc. Soc. Exp. Biol.* **92:** 253. 1956.
25. Kupperman, H. S., E. L. Wyant and A. C. Degraff: *Metabolism* **4:** 50. 1955.
26. Kepinov, L.: *Comtes rend. acad. sciences* **243:** 187. 1956.
27. Krüskemper, H. L. and G. Schulze: *Arch. exp. Path., Berlin* **227:** 161. 1956.
28. Levin, L. and R. K. Farber: *Rec. Progr. in Hormone Research,* **7:** 399. 1952.
29. Smith, M.: *Biochem. J.* **59:** 52. 1955.
30. Studer, A.: *Z. f. ges. exper. Med.* **121:** 287. 1953.
31. Tilling, W. and H. Thomann: *Z. f. d. ges. inn. Med.* **10:** 1161. 1955.
32. Erlanger, B. F., F. Borek, S. M. Beisera and S. Lieberman: *J. Biol. Chem.* **713:** 228. 1957.
33. Turner, C. D.: General Endocrinology, Philadelphia, 4/7, 1955.
34. Schneider, W. and H. Frahm: *Acta endocrinol.* **20:** 279. 1955.
35. Schmitz, E. and G. Hilgetag: *Naturwiss.* **21:** 626. 1933.
36. Schmitz, E. and J. Kühnau: *Biochem. Z.* **259:** 301. 1933.

LONG TERM EFFECTS OF DEUTERON IRRADIATION OF THE RAT PITUITARY[1]

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ABSTRACT

An investigation has been made of the long-term effect of low radiation doses to the rat pituitary using a high energy deuteron beam. The pituitaries were irradiated when the rats were 28 days old. The animals were maintained up to 27 months thereafter. A total of 138 controls and 169 treated rats were studied. Groups varying in size from 7 to 20 rats at each dose of radiation were sacrified at intervals of about 3 months.

The endocrine changes became increasingly more prominent with increasing dosage and with time. Within certain dose ranges the same terminal results were obtained, though the lower the dose the longer the period which elapsed before the maximum effect resulted.

The effect of the radiation on pituitary structure was as follows: decrease in size of all lobes; loss of demarcation between lobes; increased connective tissue in surrounding membranes and supporting framework and frequent small hemorrhages. In the anterior lobe the change effected in the shortest time and at the lowest dose was degranulation and disappearance of acidophils. Basophils were less severely damaged. This differential action of radiation on the two types of chromophils was not noted in the earlier series of experiments reported, where higher deuteron dosage was applied to the pituitary and prompt injurious action on its parenchyma resulted.

The endocrine changes in organs dependent on pituitary secretion appeared in the following sequence: reduction in adrenal weight with adrenal cortical atrophy; decrease in thyroid size and evidences of inactivity; testicular atrophy, regression of male accessory organs and failure of sperm production. The sensitivity of body growth impairment was difficult to evaluate relative to other endocrine alterations, at least at lower doses of radiation, after which the endocrine changes did not supervene at once; body size, particularly length, could not regress, whereas the weight of the endocrine organs could. The severity of all these changes following irradiation, judged by weight or histological structure or function, increased with dosage as did the speed of onset.

Adenomata were observed in pituitaries which had been irradiated with low doses. At the lowest doses, tumor incidence in irradiated pituitaries was three fold greater than in pituitaries of controls of the same age, whereas, at high doses tumor formation was suppressed. The tumors were derived from chromophobes, chiefly, but from acidophils and basophils as well; mixed-cell adenomata were also observed.

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Tumors occurred rather frequently in other endocrine organs during the second year. They were no more frequent in experimental than in control animals, and after high radiation dosage no adenomata were present in the adrenal cortices or thyroids which had become hypoplastic as a result of pituitary injury.

IT HAS been shown that the pituitary of the rat can be completely or partially destroyed by a well-localized beam of high energy deuteron particles, without evidence of serious damage to adjacent structures (1). In the first series of experiments in which such radiation was directed to the rat pituitary, massive doses were given, ranging from 3000 to 25,000 rep.[2] The observations made on groups of rats sacrificed at intervals from 4 days to 9 months post-radiation led to the following conclusions:

1. The degree of destruction of the pituitary following radiation is a function of the post-irradiation period as well as of the dose.
2. Only the highest doses, 18,900 rep and above, led to prompt and complete destruction of the pituitary. After lower doses, 6300 rep or less, the destruction was not complete even many months after irradiation (9 months).
3. There was no evidence of stimulation of the pituitary after the doses and periods of observation employed, either from morphology of the anterior pituitary or from function judged by the dependent endocrine organs.
4. There was no evidence from the dose levels employed that the pituitary recovered after the damage caused by radiation at dose levels employed. Although mitoses were observed occasionally they were abnormal.
5. No difference in sensitivity of pituitary cell types was reported, the high doses used being equally destructive to all.
6. Body weight increases and thyroid weights were impaired soon after irradiation with dose levels from 6300 to 18,900 rep, but degeneration of the testes occurred much later.

The present study is an extension of the previous work to include lower doses of deuteron radiation and longer post-irradiation intervals. The specific objectives were as follows: to search further for evidence of a differential effect of pituitary irradiation on cell types, particularly those of the anterior lobe; to determine the long-term destructive effects of low doses on anterior lobe morphology and function, and to observe any increase in the incidence of tumors in the pituitary or in other tissues of the body.

MATERIAL AND METHODS

The procedure of alignment of the pituitary in the path of the deuteron beam, the size of the aperture, the roentgenographic check of alignment,

[2] To conform with the previous publication in this series, doses are reported in rep. The new international dose unit is rad; 1 rad = 1.06 rep. = 100 erg per gm. tissue.

and the method of measurement of radiation dose and its spread have been explained in detail in the previous publication (1). The deuteron beam was collimated to conform to the shape and dimensions of the pituitary by being passed through a brass aperture of triangular shape slightly larger than the pituitary. Having once determined roentgenographically with the aid of a lead phantom placed in the pituitary site in relation to the occipito-sphenoidal synchondrosis, it was possible to direct the deuteron beam correctly by relating it to this landmark.

A group of 169 male rats of the Long-Evans strain were subjected to pituitary irradiation at 28 days of age with doses of 945, 1575, 3150 and 6300 rep. The normal controls (138 rats) were the same age and were maintained under the same conditions. As in the previous series all rats were given a complete diet which was fed *ad libitum*. Periodically the general appearance, muscle tone, body weight, tail length and any grossly evident abnormalities were recorded. Groups composed of 7 to 20 rats which had received each dosage were sacrificed 3, 7, 12, 17, 22 and 27 months after irradiation. The testes, seminal vesicles, prostate, adrenals, thyroid, thymus and pituitary were dissected, weighed and fixed for histologic study. The testes and thyroid were fixed in Bouin's fluid and embedded in paraffin and stained with hematoxylin and eosin. The adrenals were fixed in neutral formalin and sectioned while frozen to be stained, subsequently, with Sudan black for lipid. The pituitary glands were fixed in Zenker-formol, sectioned at 4 micra after nitrocellulose embedding and stained with a modified Mallory-Azan stain (2) and also according to the periodic acid Schiff (PAS) technique following Purves and Griesbach (3). Sections from 5 levels of the pituitary were studied.

RESULTS OF PITUITARY IRRADIATION ON STRUCTURE AND FUNCTION OF ENDOCRINE GLANDS

The effects of irradiation of the pituitary were estimated both by assessment of its functional state during the life of the animal and by the morphological changes in the dependent endocrine organs, as well as by direct analysis of morphological changes in the pituitary. Changes in growth hormone secretion were estimated from measurements of gain in body weight and dimensions. The effect on skeletal growth, as indicated by the average increase in tail length, is shown in Figure 1. (Data from the preceding series is included for purposes of comparison.) The increase in dimensions was not affected by 945 rep, but at dose levels of 1575 rep and above, tail growth began to lag behind that in normal rats, the retardation occurring sooner and being progressively greater at higher doses.

In order to evaluate the dose-effect relationship the data were analyzed as follows. It was assumed that that part of the increase in tail length which is dependent on pituitary function is the difference between the tail length of normal rats and that of surgically hypophysectomized animals. The tail length increase, 22 months post irradi-

ation, of groups receiving the various doses, is plotted as per cent of normal in Figure 3. It can be seen from the figure that pituitary irradiation resulted in an impairment of tail growth increment which was exponential.

Let:

l_D = tail length increase of radiated rats receiving dose D
l_h = tail length increase of surgically hypophysectomized controls
l_n = tail length increase of normal rats
D_{50} = dose necessary to reduce to 50% the pituitary dependent tail growth (difference between tail length of intact and hypophysectomized controls)
e = base of the natural system of logarithms

Then the percent change in tail increment is as follows:

$$\phi_T = 100\,\frac{l_D - l_h}{l_n - l_h} = 100e^{-0.693D/D_{50}} \qquad D_{50} \approx 5{,}500 \text{ rep}$$

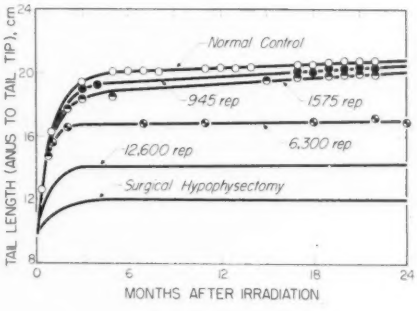

FIG. 1. Effect of pituitary deuteron irradiation on tail length of male rats. (Results from dose of 12,600 rep are interpolated (1).)

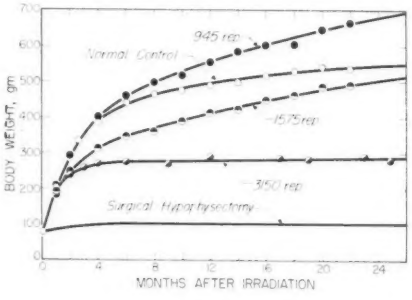

FIG. 2. Body weight of rats following various doses of pituitary irradiation. After 945 rep a portion of the rats became obese during the second year.

The average increases in body weight after pituitary irradiation as a function of time are plotted in Figure 2. Doses of 1575 rep resulted in slowing of body weight increase but not in stasis. At 3150 rep marked decrease in rate of weight gain occurred within 1 month. The higher the dose the more rapidly stasis followed, but at no dose did growth stasis follow as promptly as after surgical hypophysectomy.

When M_D is the body mass of rats having received a dose D, M_h the mass of hypophysectomized rats, and M_n the mass of normal controls, then:

$$\phi_m = 100\,\frac{M_D - M_h}{M_n - M_h} \qquad D_{50} \approx 5500 \text{ rep.}$$

Comparison of ϕ_t and ϕ_m in Figure 3 indicates the essential similarity of action of pituitary irradiation on tail length and body mass, when doses above 3000 rep were given. The dose required for reduction of post-irradiation growth to half normal is about 5500 rep (D_{50}) by each criterion, and the dose-effect relationship is a negative exponential.

At the lowest dose, 945 rep, the body weight failed to reach a plateau,

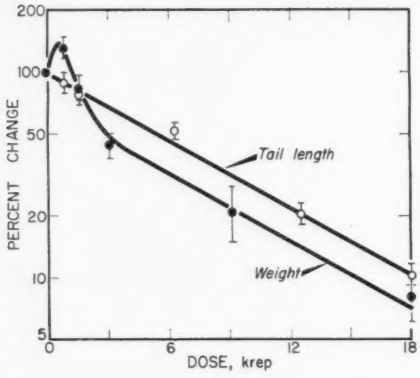

FIG. 3. Dose dependence of tail length and body weight (ϕ_l and ϕ_m) as function of deuteron dose 22 months after irradiation. Note the increase of body weight due to fat gain after the lowest dose.

but continued to increase with time. This lowest dose not only did not retard skeletal or body weight increase; instead, body weight eventually exceeded normal due to accumulation of fat. The development of obesity was noted during the first year but became definite in the 2nd year. The late appearance of adiposity did not occur in all rats of the group; 8 of 12 weighed above the normal range. The heaviest rat attained a weight of 8.00 gm., a weight 300 gm. greater than the average of controls.

In order to obtain evidence of progressive change in the organs under control of the pituitary, the status was evaluated by the weight and morphology at increasing times following pituitary irradiation. The average weights of the thyroid, adrenals, prostate, seminal vesicles and testes at various post-irradiation intervals are shown in Figures 4, 5, 7, 8 and 9.

The order in which the different endocrine organs reflected pituitary damage was the same, whether judged by the time interval before reduction of activity became manifest, or by the size of the dose necessary to produce atrophy within a given interval. The lowest dose, 945 rep, caused reduction only in adrenal weight; at 1575 rep the weight of the thyroid

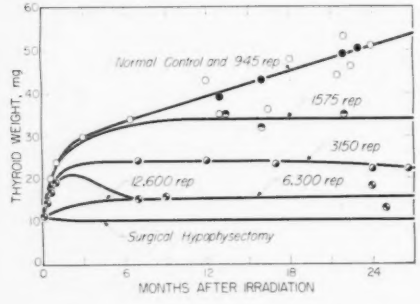

FIG. 4. Thyroid weights following pituitary deuteron irradiation.

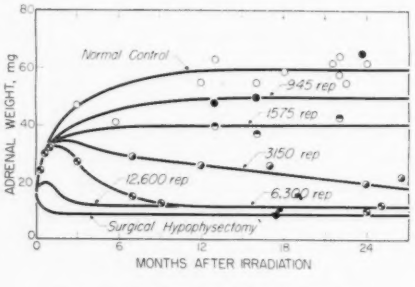

FIG. 5. Adrenal weight following pituitary deuteron irradiation.

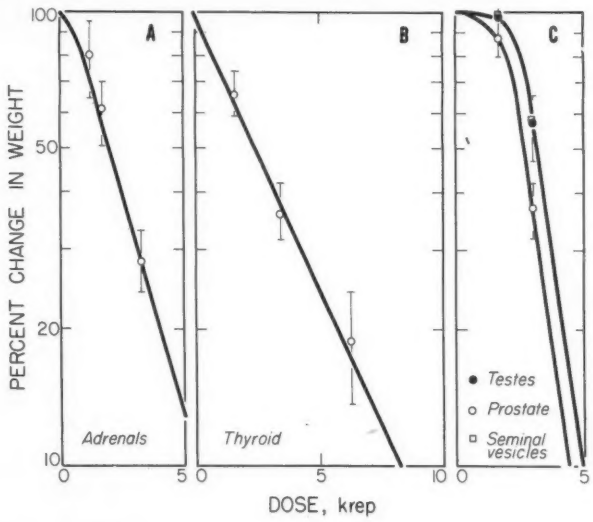

FIG. 6. A. Dose dependence of adrenal weight ($\phi_{ad.}$) 18 months after pituitary deuteron irradiation. B. Dose dependence of thyroid weight ($\phi_{th.}$) 18 months after pituitary irradiation. C. Dose dependence in weight of prostate, seminal vesicles and testes ($\phi_{pr.}$ ϕ_{sv} $\phi_{te.}$). 18 months following pituitary irradiation.

as well as of the adrenal was reduced; at 3150 the weights of all endocrine organs were reduced. Microscopic examination revealed a degree of aplasia comparable to the reduction in weight at the higher doses, eventually being comparable to the atrophy after complete surgical hypophysectomy. The order of decreasing sensitivity of the pituitary target organs to pituitary damage was therefore adrenal, thyroid, testis (seminal vesicles and prostate).

Normal function, even growth and differentiation of the pituitary target organs progressed for variable periods after irradiation, the time intervals before regression being dependent on the size of the dose given. At lower doses, 945 and 1575 rep, the testis descended into the scrotum, enlarged,

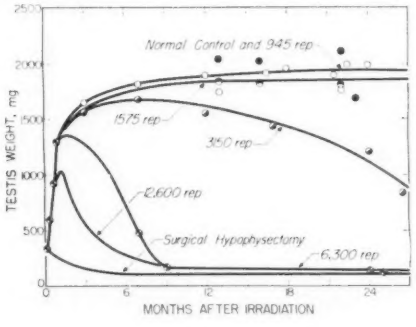

FIG. 7. Testis weight following pituitary irradiation.

and differentiated with normal sperm formation; at these low doses sperm were still present 2 years after irradiation. After 3150 rep the testes of half the animals had withdrawn from the scrotum within 2 years and resembled those after surgical hypophysectomy. After radiation with 6300 rep, half the testes were intra-abdominal by 10 months and all were atrophic by 2 years. Atrophy followed much more quickly after 12,600 rep; the testes of half the animals had withdrawn from the scrotum by 3 months. Testes appear to be the most sensitive of any target organ to small amounts of surviving pituitary tissue after radiation. The testis is also the most sensitive to minute fragments of pituitary left after surgical hypophysectomy.

The relative masses of thyroid and adrenals eighteen months after radiation, with respect to those of surgically hypophysectomized rats, are plotted in Figure 6, A and B.

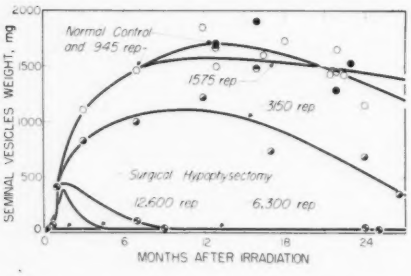

FIG. 8. Seminal vesicle weight following pituitary irradiation.

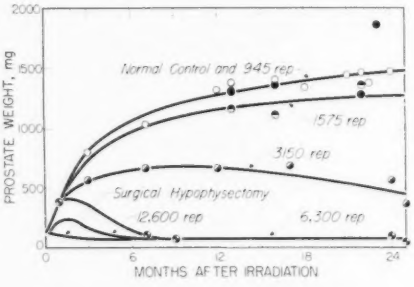

FIG. 9. Prostate weight following pituitary irradiation.

It appears that the adrenals and thyroid were much more affected by pituitary irradiation than is body weight; $D_{50\ Adrenal} \approx 2000$ rep and $D_{50\ Thyroid} \approx 2500$ rep.

At 18 or 22 months post-irradiation there was, with the exception of the lowest level, an exponential dose-effect relationship, resembling a "one hit" type radiobiological response for body mass and mass of thyroid and adrenals. However, the testes, prostate and seminal vesicles showed a different relationship. The absolute weights of testes, prostate and seminal vesicles are shown after different doses and time intervals in Figures 7, 8 and 9; the relative decrease is shown in Figure 6 C at 18–22 months; the response curve is of the "many hit" type. For the testes and dependent organs 50% normal weight was reached at approximately 3000 to 3500 rep.

MORPHOLOGICAL CHANGES IN THE PITUITARY

The weights of the pituitaries were markedly reduced after all dose levels, and the degree of atrophy progressed with dose (Table 1). After irradiation with 945 rep the pituitary weight was reduced to half normal by the end of the first year; a dose of 1575 rep resulted in a pituitary weight one-third normal by this time; after a dose of 3150 rep the pituitary weight was reduced to less than a third by 3 months; following irradiation with 6300 rep the pituitaries were a tenth the weight of pituitaries of normal rats by the 6th month, and no further reduction in weight occurred during

a longer experimental period. The weight of the irradiated pituitary is not necessarily representative of its functional capacity, since different cell types may be damaged to a different degree. Pituitaries weighing 1–2 mg. were composed largely of a framework of connective tissue, sometimes augmented by scarring and the residue of hemorrhages.

The pituitaries which were severely damaged by high doses (3150 and 6300 rep) were flattened, translucent and pale, but occasionally were hyperemic. The covering membrane was thickened and microscopic examination showed considerable scarring and heavy strands of connective tissue penetrating the parenchyma. A difficulty experienced on gross examination at autopsy in distinguishing individual lobes proved to be due to irregular invasion of cells from one lobe into neighboring lobes. After

TABLE 1. PITUITARY WEIGHTS* AFTER IRRADIATION OF THE PITUITARY WITH INCREASING DOSAGE, AFTER PROGRESSIVELY LONGER PERIODS

Dosage rep	Period after radiation in months					
	3–4	6–9	11–14	15–19	20–23	24–27
	mg.	mg.	mg.	mg.	mg.	mg.
0	10.2	10.7	12.0	12.1	12.4	13.3
945	—	—	5.9	6.5	8.4	—
1575	—	—	3.9	3.8	4.1	—
3150	2.8	1.9	1.4	1.8	1.3	1.4
6300	—	1.2	1.4	—	—	0.9

* Pituitaries with grossly discernible abnormalities were eliminated in preparing these averages.

the lower doses (945 and 1575 rep) these changes were not so evident though other gross abnormalities such as tumors, cysts, and generalized hemorrhages were more frequent. None of the latter abnormalities were observed during study of 79 pituitaries irradiated with 3150 or 6300 rep whereas 16 of 39 pituitaries showed these changes after 945 rep and 10 of 35 pituitaries showed them after 1575 rep; they appeared in only 7 of the 129 control pituitaries.

The microscopic alterations in the parenchyma of the anterior lobe after the highest dose, 6300 rep, were advanced by 6 months. Normal acidophils had practically disappeared from the anterior lobe; those which could still be distinguished were almost completely degranulated or were atypical in size and nuclear morphology. The basophils, although reduced in number and partially degranulated, were less damaged; small groups could still be found in about half the glands confined to the gonadotrophic basophil zone. Such basophils were often abnormal, and showed vacuolation either of the type present after castration or after thyroidectomy. In the intermediate lobe the chief changes were loss of the usual uniform distribution of cells and the great variability in cell size and staining reaction. The posterior lobe was the least affected; however, considerable irregularity

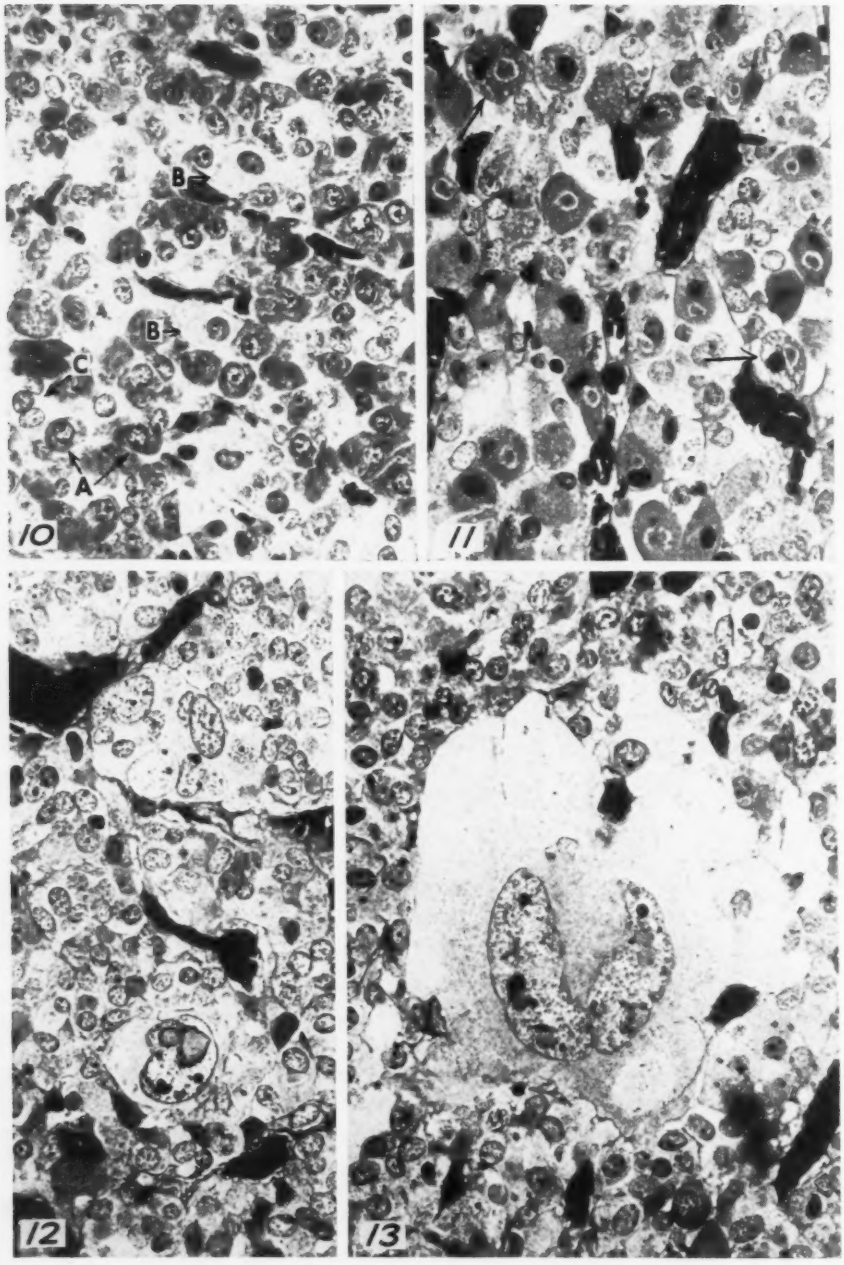

FIG. 10. Anterior pituitary of 13 mo. old control, showing normal appearance and distribution of (A) acidophils; (B) basophils; and (C) chromophobes. Mallory-Azan stain, ×500.

in cytoplasmic and nuclear size and shape was present (Fig. 17). Small localized hemorrhages were observed in all lobes. All the changes described were marked by 6 months, and maximal by 9 months; no further degeneration and no evidence of regeneration was detected up to 2 years post-irradiation.

After irradiation with 3150 rep the acidophils could still be recognized after 3 months. In the period from 6 to 24 months the changes, particularly in the acidophils, were progressive and by the end of this period many glands were indistinguishable from those which had received the higher dosage. In some glands the basophils were not decreased in numbers or extensively degranulated, regardless of the length of post-irradiation period, but had come to resemble closely the basophils of rat pituitaries after gonadectomy or after thyroidectomy. The changes in the intermediate or posterior lobes were similar to those observed after higher doses.

The pituitaries subjected to 1575 rep showed similar morphological changes but they appeared more slowly; the changes in acidophils found after 12 months were similar to those recorded for glands 3 months following 3150 rep. Basophils could be found throughout the majority of the glands; many were enlarged and showed nuclear changes and cytoplasmic vacuolation resembling those after castration or thyroidectomy (Figs. 10 and 11). Pituitaries irradiated with this dose showed no further morphological changes after 18 to 24 months, except that the acidophils virtually disappeared. Mitoses were increased at this dose and occurred in all lobes and in all cell types including chromophobes. The changes in the intermediate and posterior lobes were similar to those after 31.50 rep.

After irradiation of the pituitary with 945 rep the parenchymal elements in all pituitary lobes showed the same type of alterations as previously observed but the changes appeared more slowly. The acidophils were decreased in number and degranulated although to a lesser degree than in the preceding groups. The number of basophils remained within normal limits or appeared to be increased. Many of the basophils were abnormal showing various types of cytoplasmic vacuolation, pyknotic nuclei and peripheral clumping of granular material.

FIG. 11. Anterior pituitary 13 mo. following irradiation with 1575 rep. Acidophils had virtually disappeared whereas basophils were increased considerably in number and size and had a prominent Golgi apparatus. Some pyknosis of nuclei, and various types of cytoplasmic vacuolation were present (arrows). M.-A., ×500.

FIG. 12 and 13. Anterior pituitary 13 and 15 mo. following irradiation with 945 rep, showing pre-adenomatous changes. Clusters of atypical chromophobes, which sometimes reached gigantic proportions, were present. Note that acidophils were virtually absent. M.-A., ×500.

INCIDENCE OF TUMORS

Adenomatous Changes in Pituitaries

Adenomatous transformation occurred frequently in the pituitaries of the normal rats during the second year of the experiment. Gross abnormalities were noted at autopsy in 6 of the 116 control pituitaries examined.

TABLE 2. INCIDENCE OF TUMORS IN ENDOCRINE ORGANS DURING SECOND YEAR AFTER IRRADIATION OF THE PITUITARY

	Dosage rep	Total number of rats	Incidence of tumors		Conclusion
			No. of rats	Per cent	
Pituitary		116	37	32	No adenomas at high levels; at low levels more frequent than in controls and number per gland greater
	945	39	37	95	
	1575	32	21	65	
	3150	37	1	7	
	6300	6	0	0	
Adrenal cortex		125	35	28	Small, frequently multiple, tumors in controls and in experimental rats, except after high doses
	945	39	9	23	
	1575	41	5	10	
	3150	51	10	20	
	6300	12	1	8	
Thyroid		125	33	26	Multiple adenomas were frequent, except in glands where hypoplasia from high radiation dosage had occurred
	945	39	15	40	
	1575	41	7	16	
	3150	51	14	27	
	6300	12	2	17	
Adrenal medulla		125	10	10	Adenomas rare in all groups; none observed after high levels of radiation
	945	39	2	5	
	1575	41	1	2	
	3150	51	0	0	
	6300	12	0	0	
Parathyroid		125	5	4	Incidence low and no increase after radiation
	945	39	0	0	
	1575	41	0	0	
	3150	51	2	4	
	6300	12	1	8	
Reproductive system		125	2	2	No increase in incidence from radiation
	945	39	0	0	
	1575	41	0	0	
	3150	51	1	2	
	6300	12	0	0	

Tumors were first observed after maintenance for 16 months, and pituitary tumors were found histologically in 32% of all controls sacrificed between 16 and 24 months (Table 2). In glands which had been subjected to the higher doses of deuteron radiation, 3150 to 6300 rep, and in which severe damage to the parenchyma occurred promptly, no gross abnormalities were noted on removal of the gland and histological evidence of tumor formation was observed in only one gland. In the groups receiving the lower doses of radiation gross abnormalities were noted at autopsy in 12 of 39 pituitaries in the group irradiated with 945 rep and in 4 of the 32 irradiated

with 1575 rep. The total number of tumors and the rate of appearance were both greater in the groups receiving these two lowest levels, than in controls; after 1575 rep tumors appeared by 13 months and the incidence rose with time until between 16 and 24 months more than half (65%) of the pituitaries had developed tumors. Following 945 rep adenomatous changes were observed in 75% of the animals sacrificed after 13 months, and by the end of the second year adenomatous transformations had developed in almost all the pituitaries (95%). The tumors in the irradiated pituitaries were frequently multiple, more so than in controls, 3.1 were found per gland by 24 months compared with 1.6 in controls (Table 3).

The adenomatous changes occurred most frequently in the anterior lobe. The tumors were usually microscopic, less frequently macroscopic. Pituitaries enlarged by such tumors attained weights of 40 to 327 mg. and dimensions up to 11×7×7 mm. Most of the tumors were clearly outlined, though some were poorly defined. Some were hyperemic, or even hemorrhagic. The large number of macrophages containing pigments derived from hemoglobin bore witness to the long period during which hemorrhages had been occurring.

TABLE 3. CELLS OF ORIGIN OF TUMORS PRESENT IN PITUITARIES DURING SECOND YEAR AFTER IRRADIATION

Dosage	No. of glands having tumors	Anterior lobe				Intermediate lobe cells	Tumors	
		Acidophils	Basophils	Chromophobes	Chromophobes & acidophils		Total No.	Av. per gland
	37	1	27	30	1	0	59	1.6
945	37	16	29	50	14	6	115	3.1
1575	21	13	7	30	4	5	59	2.8
3150	1	0	0	1	0	0	1	1.0
6300	0	0	0	0	0	0	0	0

Adenomata derived from each of the three cell types were present, also tumors derived from mixed cell types (Table 3). The most dramatic increase took place in the acidophilic and in mixed acidophilic and chromophobic tumor classes; the former increased 16 fold in the 945 rep group. The incidence of tumors derived from basophils or chromophobes did not increase so markedly above that in controls. Intermediate lobe tumors appeared in rats irradiated with low doses whereas such tumors were absent in the controls.

The cells of the adenomatous areas tended to be uniform in size and frequently smaller than the corresponding normal cell type. In some the cells were larger than normal, in which case the shape and inner structure of both cytoplasm and nucleus were frequently distorted, and the staining characteristics of the cytoplasm changed, due both to depletion of the

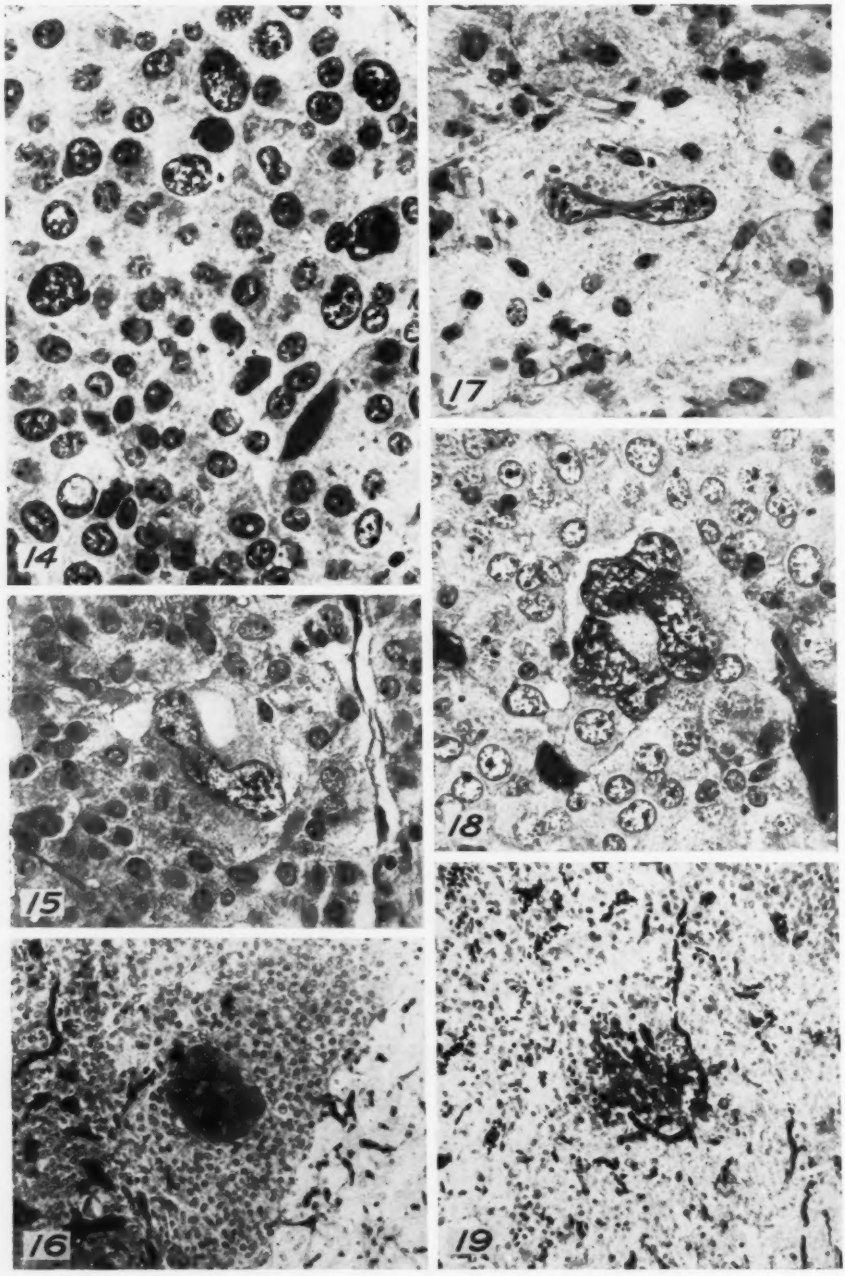

FIG. 14. Pre-adenomatous changes in the intermediate lobe, 23 mo. following irradiation with 1575 rep. Note considerable variations in cellular size and pronounced nuclear changes. M.-A., ×500.

granular content and to vacuolation of the cytoplasm. The number of mitotic divisions was notably increased in the adenomata and mitotic figures were often abnormal.

Tumors derived from chromophobes were the largest, as well as most common, and they were the only tumors in which hemorrhages were observed. Mitotic divisions were also more frequent in the chromophobic tumors. The cells did not stain intensely, and were usually not clearly outlined. In a few tumors the cells were irregularly enlarged; some attained gigantic dimensions. The outlines of the nuclei in these large cells were distorted by budding or folding of the nuclear membranes (Fig. 18) and nucleoli were prominent. The cytoplasmic outlines were more clearly defined than in the usual chromophobe.

Small PAS-positive granules were present in some of the tumors designated as chromophobe tumors, suggesting that these tumors might more properly have been grouped with basophilic tumors. Such tumors were usually smaller and less definitely outlined, and no hemorrhagic areas were present. The outlines of the cells designated here as "basophilic chromophobes" were more clearly defined than in typical chromophobic tumors. The cell size was usually smaller than that in normal basophils, and the cytoplasm stained purple rather than blue with Mallory-Azan. The nuclei were compact and polychromatic and the negative image of the Golgi apparatus was smaller than in normal basophils. A small number of the tumors was composed of cells clearly identifiable as basophils (Figs. 22 and 23).

It was also possible on the basis of staining criteria to identify some of the chromophobic tumors as composed of "acidophilic chromophobes." Tumors composed of clearly defined acidophils were the least common. Such tumors were usually small, and though not sharply outlined, were distinguishable with Mallory-Azan stain from the surrounding parenchyma by the presence of deep red or brilliant yellow stained granules (Figs. 19

FIG. 15. Gigantic cell with atypical nucleus in pre-adenomatous area of the intermediate lobe, 13 mo. following 945 rep. M.-A., ×500.

FIG. 16. Small intermediate lobe tumor (118 μ in largest diameter). The tumor consisted of large basophilic cells. These cells also gave an intense PAS reaction. M-A., ×150.

FIG. 17. Gigantic cell showing atypical nucleus from a pre-adenomatous area in the posterior lobe, 6 mo. following irradiation with 6300 rep. M.-A., ×500.

FIG. 18. Central area of chromophobic tumor in the anterior pituitary 22 mo. following 945 rep, showing enlarged nuclei with prominent nucleoli, small localized hemorrhage, and gigantic cell. Note the similarity of the atypical cells found in various parts of pituitary (compare with Figs. 12, 13, 15, 17, 18). M.-A., ×500.

FIG. 19. Small, indefinitely outlined tumor (160μ in diameter) which consisted of a cluster of brilliantly stained acidophils of varying size. Some of the acidophils were 3 times larger than normal. M.A., ×150.

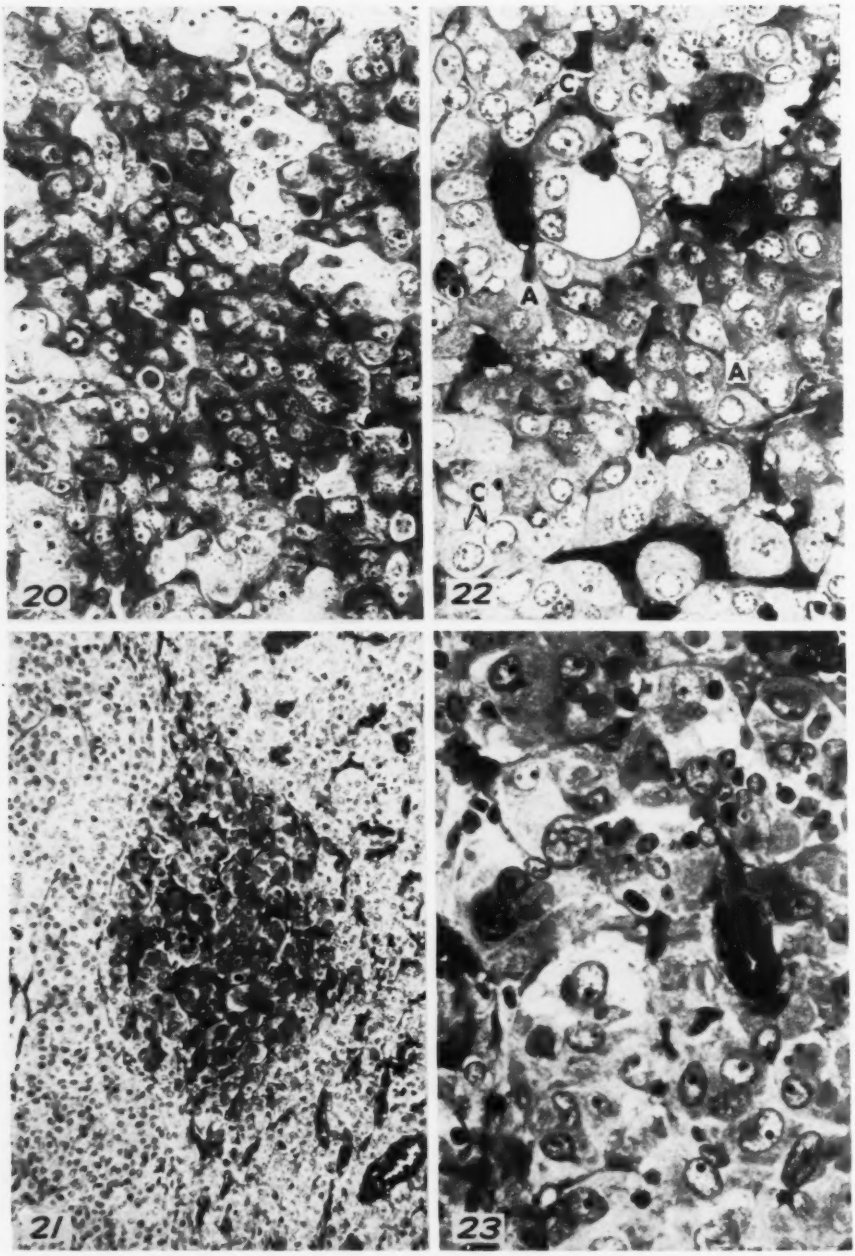

FIG. 20. Medium size tumor in the anterior pituitary 15 mo. following irradiation with 945 rep, consisting of heavily granulated and intensely stained acidophils. M.-A., ×500.

and 20). A few tumors were composed of both chromophobes and acidophils. Such areas of adenomatous change were often small and of low vascularity (Fig. 21).

In addition to such relatively circumscribed areas of adenomatous transformation in the anterior pituitaries of rats sacrificed between 12 and 24 months, clusters of parenchymal cells which appeared changed were observed in widely scattered areas. The cells in these clumps were sometimes enlarged, atypical in morphology and frequently in mitosis (Figs. 12 and 13). Such areas were considered pre-adenomatous.

Although most tumors occurred in the pars anterior, there were localized areas in the pars intermedia characterized by pronounced hypertrophy and altered morphology of the parenchymal cells (Figs. 14, 15 and 16). Both the nucleus and cytoplasm of individual cells were sometimes many times normal size. The changed cells in such areas were often arranged in cords, or formed acini with accumulation of fluid between the cells. Mitotic figures were found in such areas.

Tumor Incidence in Other Endocrine Organs

Multiple localized foci of adenomatous transformation were present in the adrenal cortex and in the thyroid of rats sacrificed during the second year (Table 2). Tumors were rare in the adrenal medulla, though a few pheochromocytomata were present both in controls and in groups receiving the low radiation to the pituitary gland. The incidence of adenomata of the parathyroid was low both in controls and in experimental groups. No adenomatous transformations were observed in the reproductive tract, with the exception of one testicular Leydig cell tumor in an irradiated group, and two small lesions resembling chorionic tissue in close relationship to the epididymis in a control group.

Incidence of Tumors in Non-Endocrine Organs

All rats maintained up to 26 months post-irradiation were examined carefully for presence of tumors in any part of the body, and any ab-

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FIG. 21. Typical, clearly outlined, small tumor (420 micra) in anterior pituitary, 22 mo. after irradiation with 945 rep. It consisted of basophils, quite uniform in appearance, showing strong PAS reaction. PAS, ×150.

FIG. 22. Central portion of a mixed-cell tumor (composed of chromophobes and acidophils) in the anterior pituitary, 23 mo. after irradiation with 945 rep. Note enlarged vesicular nuclei of chromophobes (C) and clusters of partially degranulated acidophils (A). M.-A., ×500.

FIG. 23. Central portion of a medium size basophilic tumor in the anterior pituitary, 23 mo. after irradiation with 945 rep. Note considerable variation in cell and nuclear size. In contrast with the basophils of the tumor depicted in the preceding figure, cells were irregular in size and some showed vacuolation of cytoplasm and nuclei. M.-A., ×500.

normalities detected were analyzed histologically (Table 4). The tumors were found chiefly between the ages of 12 and 24 months. Lymphosarcomas were the most common lesions, many of which appeared to originate in the lung. The incidence may have been somewhat lower in the irradiated groups. The few tumors of the pancreas observed were chiefly due to direct extension and invasion of lymphosarcomas arising in mesenteric lymph nodes (though one was probably of islet cell origin). There was no evidence of extension of lymphosarcomata through the blood channels. In one animal in which no localized lymphosarcomatous lesion was detected, lymphoid cells were present in large numbers in blood vessels of all organs ex-

TABLE 4. INCIDENCE OF TUMORS IN NON-ENDOCRINE ORGANS IN RATS SURVIVING MORE THAN ONE YEAR AFTER PITUITARY IRRADIATION

Type of tumors	Dosage rep	Total no. of rats	Tumors present	
			Number	Per cent
Lung lymphosarcomas	0	138	30	21
	945	39	3	8
	1575	42	2	5
	3150	65	7	10
	6300	19	1	5
Miscellaneous tumors (chiefly lymphosarcomas of mesenteric nodes)	0	138	11	8
	945	39	1	3
	1575	42	4	9
	3150	65	6	9
	6350	19	0	0

amined and the interpretation of lymphoid leukemia was made. Isolated instances were observed in control and irradiated rats of adenomatous or fibro-adenomatous transformation in the pancreas, parotid and mammary gland.

DISCUSSION

The incidence of tumors in pituitaries was greater in groups which had received the low doses of radiation than in controls, whereas at higher doses of radiation the appearance of tumors was virtually suppressed. Other abnormalities of the pituitary such as cysts and hemorrhages were also encountered more frequently after low radiation doses than in controls. The weights of the pituitaries of rats which received the low radiation doses increased slightly in the older groups, as can be seen in Table 1, though in preparing these averages pituitaries were eliminated which showed gross abnormalities. The increased incidence of pre-adenomatous and the multiple small areas of adenomatous change, together with the occurrence of small cysts and hemorrhages, offer a more probable explanation of the increase in weight of these pituitaries than does recovery or regeneration late after irradiation.

The number of tumors in other endocrine organs of the irradiated rats in no case exceeded those present in similar organs of controls of the same

age; it is of interest to note, however, that no tumors were detected in pituitary-dependent endocrine glands, such as adrenal cortex and thyroid, which had become aplastic secondary to the destructive action of high deuteron irradiation of the pituitary. This is in keeping with the reduced incidence of tumors reported after surgical hypophysectomy (4).

The greater radiation sensitivity of the pituitary acidophils than of basophils described here was not noted in the earlier series, probably due to the subjection of the pituitary to higher radiation dosage which was followed by more prompt, serious damage to pituitary parenchyma (1, 5, 6, 7). This greater resistance of pituitary basophils was reflected in the status of endocrine function in the irradiated animals. Evidence for diminished hormone secretion from the anterior pituitary appeared in the dependent endocrine functions in the following sequence, as the dose or time interval increased: adrenal atrophy, with decreased cortical width, thyroid atrophy, slowed body growth and finally atrophy of the testes and dependent accessory sex organs. Sperm production was lost only under extremes of dosage and post-irradiation interval, when weight of the testis had been reduced to less than half normal. This persistence of testicular function can perhaps be attributed to the persistence of small amounts of pituitary tissue at high doses and the relatively great resistance of pituitary basophils even at high radiation dosages and over prolonged post-irradiation periods. The resistance of pituitary basophils to irradiation was evidenced to a lesser degree by the status of the thyroids.

Adiposity occurred during the second year post-irradiation after the lowest dose of radiation (945 rep). As noted previously this increase in weight in many rats of this group far exceeded the gradual weight gain in old normal rats. If it were assumed that the adopisity observed was due to radiation injury to the hypothalamus, it would have to be assumed that this was an effect manifesting itself only at the lowest doses of radiation and only after a prolonged period.

REFERENCES

1. Tobias, C. A., D. C. Van Dyke, M. E. Simpson, H. O. Anger, R. L. Huff and A. A. Koneff: *Amer. J. Roent., Ra. Ther. and Nuc. Med.* **72**: 1. 1954.
2. Koneff, A. A.: *Stain Technol.* **13**: 49. 1938.
3. Purves, H. D. and W. E. Griesbach: *Endocrinology* **49**: 244. 1951.
4. Moon, H. D. and M. E. Simpson: *Cancer Research* **15**: 403. 1955.
5. Vogel, F. S., C. G. Hoak, J. C. Sloper and W. Haymaker: *J. Neuropath. and Exp. Neurology* **17**: 138. 1958.
6. Haymaker, W., W. J. H. Nauta, J. C. Sloper, G. Laquer, Col. J. E. Pickering, USAF, Capt. F. S. Vogel, USAF (MC): *J. Neuropath. and Exp. Neurology* **17**: 12. 1958.
7. Allen, B. M., O. A. Schjeide, R. T. Piccirillo and G. Kowitz: *J. Exp. Zool.* **124**: 131. 1953.

EFFECT OF ETHIONINE ON THE CORTISONE-EVOKED STIMULATION OF TRYPTOPHAN PEROXIDASE-OXIDASE ACTIVITY[1]

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ABSTRACT

The activity of tryptophan peroxidase-oxidase was studied in rat liver following intraperitoneal injections of cortisone, ethionine, and methionine. Cortisone elicited the expected increase in activity, a dose of 50 mg. per kg. evoking a five-fold stimulation. Ethionine and methionine (0.77 mM.), on the other hand, did not alter the activity of this enzyme. Administration of ethionine with cortisone greatly reduced the stimulatory effect of the hormone. Coadministration of methionine with ethionine (both at a dosage of 0.77 mM.) restored the ability of cortisone to stimulate tryptophan peroxidase-oxidase activity. These results are taken to support the hypothesis that a *de novo* synthesis of this enzyme, believed to occur as a result of tryptophan administration, may also occur following the administration of cortisone.

THE activity of the enzyme tryptophan peroxidase-oxidase,[2] which catalyzes the conversion of L-tryptophan to L-formylkynurenine, has been shown to be increased in response to (a) administration of large doses of its substrate, tryptophan, and (b) administration of the adrenal corticoids, cortisone and hydrocortisone, or any agent which will elicit their secretion (1–4). Evidence has accumulated indicating that the stimulatory effect manifested by tryptophan is the result of an induced *de novo* synthesis of the enzyme (1, 5–9). Among the evidence in favor of this mammalian adaptation phenomenon is the demonstration by Lee and Williams (5) that ethionine inhibited the increase in TPO activity occurring as a result of tryptophan injection and that this inhibitory effect of ethionine could be at least partially reversed by the simultaneous administration of equimolar concentrations of methionine. Their results were interpreted to mean that ethionine suppresses the adaptive response of TPO to tryptophan through its well known inhibition of methionine incorporation into protein (10), thus interfering with the *de novo* synthesis of the enzyme.

Although the stimulation of TPO activity by adrenal corticoids has been observed in several laboratories (3, 8, 11), the mechanism of this effect remains to be elucidated. This investigation was undertaken to de-

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[2] Tryptophan peroxidase-oxidase is hereafter abbreviated as TPO.

termine the effect of ethionine on the corticoid-evoked stimulation in the belief that some insight into this hormonal response might be obtained through use of the ethionine-methionine interrelationship.

METHODS AND MATERIALS

Male Sprague-Dawley albino rats weighing approximately 200 grams were used. The rats were maintained on Purina laboratory chow fed *ad libitum;* however, only animals with matched food intakes were utilized in order to preclude possible effects due to variation in tryptophan consumption. Five hours prior to sacrifice, each animal was injected intraperitoneally with one of the following groups of materials: (1) 5 ml. of physiological saline, (2) L-methionine, 0.77 mM., (3) L-ethionine, 0.77 mM., (4) cortisone (saline suspension) 50 mg. per kg., (5) cortisone, 50 mg. per kg. and ethionine, 0.77 mM., (6) cortisone, 50 mg. per kg., ethionine, 0.77 mM. and methionine, 0.77 mM. Doses of ethionine and methionine used in this investigation conform to those of Lee and Williams (5) while the level of cortisone administered corresponds to the maximum amount given by Thomson and Mikuta during their dose-response studies (11).

Following decapitation, ten per cent liver homogenates of each animal were assayed for TPO activity by the method of Knox (12). Suitable aliquots of each homogenate were dialyzed against distilled water at 5° C for 36 hours prior to triplicate Kjeldahl nitrogen determinations by the method of Parnas and Wagner (13). In calculating nitrogen content, it was necessary to correct for volume changes which took place during dialysis of the homogenates. Specific activity of TPO is expressed as micromoles of kynurenine formed per hour per mg. of homogenate nitrogen.

The L-methionine, L-ethionine and cortisone used were obtained from the California Corporation for Biochemical Research.

RESULTS AND DISCUSSION

Rat liver TPO activities, five hours after injections of methionine, ethionine, cortisone, and combinations thereof, are presented in Table 1. Neither methionine ($P > 0.1$) nor ethionine ($P > 0.02$) significantly[3] altered the level of hepatic TPO activity from that found in the saline control animal. Cortisone administration, on the other hand, increased the activity of this enzyme five- to six-fold ($P < 0.001$), an increase which was somewhat greater than that obtained by other investigators (3, 11). The concomitant administration of 0.77 mM. of ethionine and cortisone resulted in a significantly lower ($P < 0.001$) TPO activity than that observed with cortisone alone. Injection of equimolar amounts of methionine and ethionine with cortisone restored the elevated TPO activity characteristic of rats treated only with cortisone ($P < 0.001$). Although the mean value was slightly higher, the enzyme activity of rats given cortisone, ethionine, and methionine did not differ significantly from the activity of the cortisone-treated animals ($P > 0.05$).

If, in the absence of evidence to the contrary, the usually accepted mode of ethionine inhibition is assumed to be valid under the conditions of this investigation, the following interpretation of the results obtained is possi-

[3] Accepting $P < 0.01$ as significant. Probability levels were calculated by means of the Student's "t" Test.

ble. Cortisone may effect an increased TPO activity in the liver of the intact rat by means of a mechanism which involves stimulation of the *de novo* synthesis of the enzymatically active protein molecule from amino acid precursors available at the site of the "enzyme-forming system." The presence of ethionine, as administered, inhibits optimal incorporation of essential methionine into the enzyme protein and thereby partially suppresses the production of active TPO in response to cortisone. Methionine, if present in suitable quantities, acts to "reverse" the ethionine inhibition of TPO synthesis, presumably by competing with ethionine at the site of enzyme formation, thereby permitting the expected rise in TPO activity in

TABLE 1. RAT LIVER TRYPTOPHAN PEROXIDASE-OXIDASE ACTIVITY FOLLOWING ADMINISTRATION OF METHIONINE, ETHIONINE, AND CORTISONE

Group	No. of Rats	Materials Administered	TPO Activity* μM. kynurenine Mg. N hour
I	10	Saline (controls)	0.0810 ± 0.0076
II	9	Methionine, 0.77 mM.	0.0991 ± 0.0077
III	9	Ethionine, 0.77 mM.	0.0528 ± 0.0087
IV	10	Cortisone, 50 mg./kg.	0.4415 ± 0.0386
V	9	Cortisone, 50 mg./kg. Ethionine, 0.77 mM.	0.2161 ± 0.0123
VI	10	Cortisone, 50 mg./kg. Ethionine, 0.77 mM. Methionine, 0.77 mM.	0.5532 ± 0.0509

* Mean Values and Standard Error of the Mean.

response to the hormone. In the absence of cortisone, ethionine might not be expected to have a significant effect on the constititutive enzyme. Thus the results of this investigation lend support to the previously proposed (3) existence of a common step in the "hormone-induced" and "substrate-induced" mechanisms of TPO stimulation: viz. an increased *de novo* synthesis of the enzyme.

In certain *in vitro* studies utilizing rat liver slices (14) and "cell-free" preparations (3, 8), in which tryptophan stimulation of TPO activity was shown to occur, cortisone had no significant effect. Such evidence indicates that, although both the tryptophan and corticoid stimulations may involve *de novo* enzyme synthesis, the modes of action are probably independent. The mechanisms by which tryptophan and the corticoids initiate *de novo* TPO synthesis remain to be elucidated.

REFERENCES

1. KNOX, W. E. AND A. H. MEHLER: *Science* **113**: 237. 1951.
2. KNOX, W. E.: *Brit. J. Exptl. Pathol.* **32**: 462. 1951.

3. KNOX, W. E. AND V. H. AUERBACH: *J. Biol. Chem.* **214:** 307. 1955.
4. LEE, N. D.: *J. Biol. Chem.* **219:** 211. 1956.
5. LEE, N. D. AND R. H. WILLIAMS: *Biochim. et Biophys. Acta* **9:** 698. 1952.
6. LEE, N. D. AND R. H. WILLIAMS: *J. Biol. Chem.* **204:** 477. 1953.
7. GROS, P., G. P. TALWAR AND J. COURSAGET: *Bull. Soc. Chim. Biol.* **36:** 1569. 1954.
8. GORDON, M. W. AND I. J. RYDZIEL: *Abstracts Amer. Chem. Soc.* April: 28C. 1958.
9. PRICE, JR., J. B. AND L. S. DIETRICH: *J. Biol. Chem.* **227:** 633. 1957.
10. SIMPSON, M. V., E. FARBER AND H. TARVER: *J. Biol. Chem.* **182:** 81. 1950.
11. THOMSON, J. F. AND E. T. MIKUTA: *Endocrinology* **55:** 232. 1954.
12. KNOX, W. E.: *Methods in Enzymology:* Colowick and Kaplan, Editors, Academic Press, Inc. New York. 2: 242. 1955.
13. NIEDERL, J. B. AND V. NIEDERL: *Micromethods of Quantitative Organic Elementary Analysis*, John Wiley and Sons, Inc. New York. 51. 1938.
14. CIVEN, M. AND W. E. KNOX: *Federation Proc.* **16:** 165. 1957.

EFFECT OF HORMONES UPON REGRESSION OF MUSCLE ATROPHY OF NERVOUS ORIGIN

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ABSTRACT

The effect of various hormones upon the regression of muscle atrophy of nervous origin has been studied in young rats. Unilateral nerve lesion was performed by crushing the obturator, sciatic and femoral nerves intrapelvically—a procedure which resulted in marked atrophy of the triceps surae muscle. Subsequent to complete functional recovery, treatment with hormones was initiated to see whether it would alter the course of the regression of the muscular atrophy.

STH, methyltestosterone, estradiol and thyroxin promote the rate of regression subsequent to reinnervation. Treatment with cortisol or 17-ethyl-19-nortestosterone, at the high dose levels used, exerts an opposite effect, while desoxycorticosterone has no influence.

From the results obtained in these experiments it can be concluded that, under certain experimental conditions, catabolic hormones, e.g., thyroxin and estradiol, can promote a circumscribed anabolic process, such as regression of muscle atrophy.

WHEN a skeletal muscle is deprived of its lower motor neuron, a process usually spoken of as denervation, a number of characteristic changes in the muscle take place (1). Such denervation is seen in many clinical conditions, most important of which are poliomyelitis and trauma to peripheral nerves. Unfortunately, both are common in normal times, but the incidence of the latter has been greatly increased in every war. Subsequent to nerve injury, under favorable conditions a functional motor reinnervation may take place when the progress of muscle atrophy is arrested and regression is initiated. But some degree of permanent atrophy is always to be anticipated after nerve injury. In spite of many new findings in this field (2–6) it is not possible, at the present time, to state precisely which factors are most important in determining the onset and rate of the development and regression of muscle atrophy. A disturbance of the trophic influence of the nervous system is generally assumed to exist (7), but the term "trophic influence" lacks physiological definition.

A review of the literature reveals many glowing reports of results obtained from treatment of post poliomyelitis, and similar conditions with local neural atrophies of the muscle tissue, by numerous and widely different forms of physical therapy (8). But it is important to point out that,

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based on the experimental results, considerable controversy exists as to the efficacy of electrical stimulation (9). Although it is possible to retard the development of muscle atrophy with induced activity, the onset is never completely prevented even though specific function is substituted by direct stimulation. Also, there is still the problem of finding a non-injurious agent which will be tolerated by the patient and will effectively retard atrophy and facilitate regeneration of muscle.

This report is concerned with experiments dealing with the effects of various hormones upon the regression of muscular atrophy of nervous origin. The experiments were carried out on the triceps surae muscle of the rat, because the extent and rate of development of muscle atrophy and of regeneration of nerves was found to run a remarkably constant course in this species (9, 10). One hind limb was paralyzed by unilateral crushing of the obturator, sciatic and femoral nerves and, thus, marked atrophy was produced. When functional recovery was complete, the degree of this neuromuscular atrophy was determined and the effect of hormones upon regression of the atrophy following reinnervation was studied.

EXPERIMENTAL MATERIAL AND TECHNIQUES

General experimental design. One hundred and ten young female Sprague-Dawley rats, with an average initial body weight of 51 gm. (range: 43–60 gm.), were subdivided into eleven equal groups. *Groups I and II* served as absolute controls. The rats of Group I were killed the day the experiment was started and were used as indicators of the normal initial values; those of Group II were killed on the last day (56th day) of the experiment to show the normal final values. *Groups III and IV* served as untreated controls in that they received no hormone treatment but were submitted to the unilateral nerve-crush procedure described below. The rats in Group III were killed on the 26th day of the experiment (when complete functional recovery was observed in all groups and hormone treatment was initiated). These animals served to show the average degree of atrophy that existed at this time. Group IV were killed at the end of the experiment to show the rate and degree of the spontaneous regression of muscle atrophy which takes place in the animals not treated with hormones. Rats in *Groups V–XI* were also submitted to the unilateral nerve crush procedure and treated as indicated in Table 2.

Throughout the period of observation, all the rats were fed exclusively on ground laboratory diet, Purina fox chow, and tap water. The food was ground, otherwise the animals would have had difficulty in eating it, as the teeth of all groups were clipped once every week—a procedure which prevented the animals from gnawing the denervated limb.

Production of muscular atrophy of nervous origin. In all rats of Groups III–XI, unilateral nerve lesion was performed under light ether anesthesia, through a suprapubic incision, on the first day of the experiment. The left obturator, sciatic and femoral nerves were intrapelvically crushed with narrow forceps, so that transparency of the nerve was produced at the site of crushing, which ensured the complete interruption of axons. In all instances, complete insensitivity and paralysis of the left limb resulted. This method of denervation leads to a most successful regeneration of the nerves (10, 11) and on the other hand, allows for the study of the regression of the muscle atrophy after reinnervation. The sensitivity and motility of the denervated limb were examined every

second day after the surgical intervention. The recovery of sensory and motor function were separately assessed in the arbitrary scale of 0 to 3; the degeneration of the crushed nerves was estimated in an index of 0–6 (sensitivity plus motility). The index of 6 served as a criterion for "complete functional recovery," i.e., the sensitivity and the voluntary movement of the affected limb were both normal.

Treatment with hormones. On the 26th day of the experiment, treatment with various hormones was initiated in Groups V-XI as indicated in Table 2 and was continued until the termination of the experiment. The hormones were always injected in 0.2 ml. of water, subcutaneously.

Cortisol acetate was administered in the form of microcrystals of hydrocortisone acetate (Schering), at the dose of 1 mg./day (Group V).

Desoxycorticosterone acetate was administered in the form of Cortate (Schering), at the dose of 1 mg./day (Group VI).

STH (somatotrophic hormone) was administered in the form of the growth hormone (U.S. National Institutes of Health), in 2 daily injections of 1 mg. (Group VII).

Estradiol, Progynon (Schering), was administered in the form of micro-crystals, at the dose of 500 μg./day (Group VIII).

Methyltestosterone, Oreton-M (Schering), was given in the form of microcrystals, at the dose of 2 mg./day (Group IX).

17-Ethyl-19-nortestosterone was administered in the form of Nilevar (G.D. Searle & Co.), at the dose of 2 mg./day (Group X).

Thyroxin obtained from the British Drug Houses, was given at the dose of 250 μg./day (Group XI).

The evaluation of the experiment. On the 56th day of the experiment (i.e., 30th day after the initiation of hormone treatment) all rats were killed with chloroform and the sciatic nerve and the triceps surae muscle were dissected. After fixation in Susa solution, the muscles were weighed on an analytical balance. The means of these weights, the final body weights and the ratio of the muscle weight to body weight are given in Table 1 and 2 together with the standard errors. The differences between the contralateral muscles are especially indicated in a separate column, in which the weight of the muscle of the limb with nerve lesion is expressed as a percentage of the weight of the corresponding muscle of the intact limb. Following the weighings, the muscles were embedded in paraffin and stained with hematoxylin-phloxine for subsequent histological study. For the morphological studies of the sciatic nerve, Marchi stain was also used.

In all groups where as judged from the muscle weights a beneficial effect of the hormone treatment was observed (Groups VII, VIII, IX, XI), the findings were confirmed by measurement of muscle fibers. The diameter of 200 muscle fibers was measured for each triceps surae muscle (100 from transverse sections and 100 from longitudinal sections), histograms were drawn and the average diameter of the muscle fibers was determined. Fiber diameters were determined on both longitudinal and transverse sections, because even a small inclination of the cutting plane could considerably change the results obtained from transverse sections only, and a greater margin of error would result in the measurement of the smaller dimensions of the atrophic muscle fibers. The accuracy of the method was checked by measuring diameters (of both transverse and longitudinal sections) of the triceps surae muscles of the initial controls (Group I). The average value obtained from transverse sections of 100 muscle fibers was practically the same as that obtained by measurement of the longitudinal sections of 100 muscle fibers (left limb 17.9 μ:18.1 μ; right limb 18.0 μ:18.2 μ). Therefore, to obtain a high degree of accuracy, measurements from transverse plus longitudinal sections were taken throughout this work. The means of the average fiber-diameter values (with standard errors) and the percentile diameter differences of the contralateral muscles, are listed in Table 3.

RESULTS

The growth of intact muscle. The results summarized in Table 1 indicate that in early life (age: 4 to 12 weeks), the ratio of muscle weight to body weight increases in the rat; the triceps surae muscle gains weight faster than does the body as a whole.

Among the animals treated with anabolic hormones, only those treated with methyltestosterone showed muscle growth that paralleled that of somatic growth (cf. Table 2, Group IX). Administration of STH, while further facilitating muscle growth, produces a decrease in the ratio of muscle weight to body weight. 17-Ethyl-19-nortestosterone as well as desoxycorticosterone, at the high dose level used here, slightly diminished the normal muscle growth and somewhat decreased the muscle/body weight ratio. This decrease was more significant in the cortisol, estradiol and thyroxin treated groups, where the hormones, by their general catabolic effect, also produced a marked inhibition of the triceps surae growth.

The regression of muscle atrophy. In evaluating this data, we must keep in mind that at the time of the initiation of the treatment with hormones the average degree of muscle atrophy was 50.6%, as judged by the control animals (Group III). This figure had risen to 71.1% by the end of the experiment in the untreated rats (Group IV).

It can be seen from the results given in Table 2 (especially from the percentile differences of the contralateral muscles) that the effect of hormones on muscle which is in a process of regeneration is different from that on intact muscle. STH, methyltestosterone, estradiol and thyroxin promote the process of regression, while cortisol, and 17-ethyl-19-nortestosterone exert an opposite effect. Only desoxycorticosterone did not influence the rate of regression of the muscular atrophy subsequent to reinnervation. Nevertheless, it is important to point out that the subnormal ratio of the muscle weight to body weight due to the nerve lesion was only positively affected in the methyltestosterone and estradiol treated rats (compare the ratio of the muscle weight to body weight of Groups VIII and IX with Group IV). Especially in those treated with methyl-testosterone, this ratio was nearly normal (cf. Group IX with Group II).

The histologic observations can be summarized as follows: On initiation of the hormone treatment (on the 26th day after nerve crush) there was a marked atrophy of the affected triceps surae muscle, with the usual reduction of the gross bulk of the muscle and the dimensions of each of its constituent fibers. At this time, no degenerative process was observed. The microscopic examinations on the 56th day (30th day after complete functional recovery) also showed atrophy, but most of the fiber diameters had increased, while some other cells had even reduced their size and a few degenerated fibers with phagocytotic processes were observed. Therefore, there was greater variability of the fiber-diameters in the affected than in

TABLE 1. CONTROL VALUES FOR NORMAL SOMATIC GROWTH AND MUSCLE GROWTH (GROUPS I AND II) AS WELL AS FOR THE SPONTANEOUS RATE OF REGRESSION OF MUSCLE ATROPHY SUBSEQUENT TO REINNERVATION (GROUPS III AND IV)

Group	Treatment	Days of experiment	Body weight (gm.)	Triceps surae weight (mg.)			Triceps surae weight as percentage of body weight‡	
				Intact	Nerve lesion	%†	Intact	Nerve lesion
I	Control (initial)	0	51 ± 1.4	254 ± 1.6	(255 ± 1.2)	100.3 ± 0.44	49.8 ± 1.42	(50.0 ± 1.56)
II	Control (final)	56th	167 ± 4.5	1123 ± 15.9	(1119 ± 16.4)	99.6 ± 0.65	67.2 ± 1.57	(67.0 ± 1.35)
III*	Nerve lesion (initial)	26th	96 ± 3.6	532 ± 11.3	265 ± 9.1	50.6 ± 1.15	55.4 ± 1.19	27.6 ± 0.92
IV	Nerve lesion (final)	56th	161 ± 7.1	1112 ± 23.2	791 ± 15.1	71.1 ± 1.10	69.0 ± 1.75	49.1 ± 1.22

* Group III was killed on the day that hormone treatment was started in Groups V–XI (cf. Table 2).
† The weight of the intact muscle is taken as 100 per cent.
‡ The muscle weights of the intact and nerve crushed sides are expressed as percentages of the final body weights

$$\left(\frac{\text{weight of muscle in mg.}}{\text{final body weight in gm.}} \cdot 100\right).$$

TABLE 2. EFFECT OF HORMONES UPON REGRESSION OF MUSCLE ATROPHY OF NERVOUS ORIGIN

Group	Treatment	Final body weight (gm.)	Triceps surae weight (mg.)			Triceps surae weight as percentage of body weight*	
			Intact	Nerve lesion	%*	Intact	Nerve lesion
V	Nerve lesion + COL-Ac	92 ± 6.1	605 ± 19.8	374 ± 7.4	61.8 ± 1.35	65.7 ± 1.36	40.6 ± 0.88
VI	Nerve lesion + DOC-Ac	166 ± 7.6	941 ± 9.4	668 ± 8.9	70.9 ± 1.46	56.6 ± 1.27	40.2 ± 1.10
VII	Nerve lesion + STH	233 ± 12.2	1342 ± 11.0	1078 ± 15.0	80.3 ± 0.98	57.5 ± 0.75	46.2 ± 1.32
VIII	Nerve lesion + Estradiol	109 ± 5.5	688 ± 7.5	587 ± 5.2	85.4 ± 1.06	63.1 ± 1.58	53.8 ± 1.09
IX	Nerve lesion + Methyltestosterone	165 ± 14.8	1147 ± 7.9	1031 ± 12.1	89.8 ± 1.25	69.5 ± 1.15	62.4 ± 1.43
X	Nerve lesion + 17-Ethyl-19-Nortestosterone	162 ± 10.9	1014 ± 15.2	689 ± 12.7	67.9 ± 0.79	62.5 ± 1.40	42.5 ± 1.26
XI	Nerve lesion + Thyroxin	131 ± 8.4	696 ± 18.1	557 ± 13.5	80.0 ± 1.21	53.1 ± 0.88	42.5 ± 1.19

* For further interpretation, see the descriptions of the corresponding columns in Table 1.

the corresponding intact muscle. The same is true also for all the hormone treated groups, which did not show any other marked histologic changes. In regard to nerve studies, at the end of the experiment no areas of degeneration in the sciatic nerve, distal from the site of crushing, were observed.

From the figures in Table 3, it can be stated that the results obtained with the muscle weight studies were, in general, confirmed by fiber-size measurements in the triceps surae muscle. The regeneration-facilitating effect of STH, however, seemed to be more evident and, on the other hand, the positive influence of thyroxin proved to be less significant, as judged by determinations of the average diameters in muscle fibers.

TABLE 3. RESULTS OF THE STUDIES ON MUSCLE-FIBER DIAMETERS

Group	Treatment	Fiber size in triceps surae (μ)		
		Intact	Nerve lesion	%*
I	Control (initial)	18.1 ± 0.47	(18.0 ± 0.55)	99.4
IV	Nerve lesion (final)	30.7 ± 0.61	20.8 ± 1.56	67.7
VII	Nerve lesion + STH	34.9 ± 0.55	29.7 ± 1.41	85.3
VIII	Nerve lesion + Estradiol	24.6 ± 0.42	20.2 ± 1.21	82.1
IX	Nerve lesion + Methyltestosterone	31.6 ± 0.56	27.3 ± 1.12	86.3
XI	Nerve lesion + Thyroxin	26.1 ± 0.36	19.9 ± 1.38	76.2

* The average fiber-diameter of the intact muscle was taken as 100%.

DISCUSSION

It is evident from the summary of our findings that among the hormones used, methyltestosterone exerted the most beneficial effects upon the regression of muscle atrophy of nervous origin. Since 17-ethyl-19-nortestosterone proved to be a highly active anabolic steroid in man (12) and its anticatabolic properties have also been experimentally demonstrated (13), we decided to compare its effect on muscular tissue with that of the relatively more virilizing methyltestosterone. Our results now indicate that at the high dose level used none of these hormones stimulate the somatic growth and their effect is different upon the intact and especially on the regenerating muscular tissue: methyltestosterone facilitates the regression of muscle atrophy to near completion, while 17-ethyl-19-nortestosterone produces a marked inhibition of this process. It seems possible that the anabolic effects of testoid compounds upon the muscular tissue depend not only upon the dose and duration of the treatment (14,15; for further literature see Ref. 16), but also on other, as yet uncertain, conditions.

The regeneration-facilitating effect of STH is also satisfactory, because based on a comparison with other observations (17, 18) it can be assumed that the decrease of the ratio of the muscle weight to body weight produced by STH in the present investigation is characteristic only during an early period of life and is, thus, conditional.

It is important to point out furthermore that estradiol, despite its gen-

eral catabolic properties, was unexpectedly found to be effective in facilitating the regression of muscle atrophy. Our experiment clearly demonstrates the enhanced regression after estradiol treatment, but as only intact female rats were used, there is no indication of the mechanism involved.

As regards the effect of thyroxin, it is worthy of mention that previous experiments demonstrated that this hormone accelerates the rate of atrophy of denervated muscle (6, 9). It is believed that thyroxin administration exerts this effect by acting as an accelerator to the catabolic processes involved in the disintegration of the cytoplasm of the denervated muscle. Therefore, the observation that thyroxin does not exert the same unbeneficial effect on the regenerating muscle is worthy of attention.

The finding that cortisol, by diminishing the normal regression process, resulted in a more marked degree of muscle atrophy between all groups may be compared to recent findings. These demonstrated not only the aggravation of the development of muscle atrophies of nervous and disuse origins (3, 6), but also the production of a circumscribed muscular atrophy (2, 4) after cortisol treatment.

In considering the present findings, it should finally be noted that by employing a nerve crush method similar to that used in these studies, we were able to demonstrate that some degree of permanent atrophy is to be anticipated after nerve injury. An experiment was performed on 8 rats with an initial body weight of 53 ± 3.4 gm. Five months after the nerve lesions were performed, the following results were observed: Final body weight, 246 ± 12.6 gm.; intact triceps surae weight, 1608 ± 10.6 mg.; contralateral muscle weight (on the side with nerve lesion), 1151 ± 14.2 mg.; degree of muscle atrophy (percentage difference), 71.5%; ratio of the intact muscle weight to body weight, 65.4%; ratio of affected muscle weight to body weight, 46.8%.

Comparison of these findings with those after STH, methyltestosterone, estradiol and thyroxin treatments shows that over a considerably short period these hormones are able to increase the regression of the muscle atrophy to above the final degree which is seen in animals not treated with hormones.

It is also of interest to note that the data obtained in these experiments allow for the conclusion that, under certain conditions, catabolic hormones (e.g., thyroxin and estradiol) can even promote a circumscribed anabolic process, such as the regression of atrophied muscle tissue.

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REFERENCES

1. TOWER, S. S.: *Physiol. Rev.* **19:** 1. 1939.
2. BAJUSZ, E.: *Canad. Rev. Biol.* 1958 (in press).
3. BAJUSZ, E.: *Canad. J. Biochem. & Physiol.* **36:** 825. 1958.
4. BAJUSZ, E.: *Naturwissenschaften.* **11:** 271. 1958.
5. FERGUSON, A. B., L. VAUGHAN AND L. WARD: *J. Bone & Joint Surg.* **39-A:** 583. 1957.
6. SELYE, H. AND E. BAJUSZ: *Am. J. Physiol.* **192:** 297. 1958.
7. HNIK, P.: *Experientia.* **12:** 279. 1956.
8. MCCARROLL, H. R.: *J.A.M.A.* **120:** 517. 1942.
9. HINES, H. M. AND G. C. KNOWLTON: Proc. Soc. Exper. Biol. & Med. **31:** 1029. 1934.
10. GUTTMANN, E.: *J. Neurol. and Psychiatry* **5:** 81. 1942.
11. LEE, F. C.: *Physiol. Rev.* **9:** 575. 1929.
12. SPENCER, H., E. BERGER, M. L. CHARLES, E. D. GOTTESMAN AND D. LASZLO: *J. Clin. Endoctrinol. & Metab.* **17:** 975. 1957.
13. SELYE, H. AND S. RENAUD: *Am. J. Med. Sc.* **235:** 1. 1958.
14. DRILL, V. A. AND F. J. SAUNDERS: In: Hormones and the Aging Process. New York Academic Press, Inc., p. 99, 1956.
15. SELYE, H. AND R. K. MISHRA: Arch. internat. Pharm. Therap. 1958 (in press).
16. SELYE, H., A. HORAVA AND G. HEUSER: Annual Report on Stress. Montreal, Acta Inc., 1951–56. Vol. 1–5.
17. SIMPSON, M. E., H. M. EVANS AND C. H. LI: *Growth.* **13:** 151. 1949.
18. BIGLAND, B. AND B. JEHRING: *J. Physiol.* **116:** 139. 1952.

EFFECT OF PROGESTERONE AND ESTRONE ON THE INCIDENCE OF CONGENITAL MALFORMATIONS DUE TO MATERNAL VITAMIN E DEFICIENCY[1]

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ABSTRACT

In a series of experiments 299 Holtzman rats were used to determine the effect of progesterone and estrone on the incidence of congenital abnormalities induced by marginal maternal vitamin E deficiency. It was observed that daily subcutaneous injection of 4 mg. of progesterone or 2 μg. of estrone, singly or in combination, from the third day of gestation till term, in addition to 2 mg. of *d,l*-alpha-tocopherol acetate supplementation on the 10th day, resulted in a reduction of the weight gain of the mother rats during gestation, the average number of implantation sites and the percentages of normal and abnormal young, with concomitant increase in the incidence of resorption. This abolition of the appearance of congenital abnormalities was further substantiated unequivocally when a single dose of 4 mg. of progesterone or 2 μg. of estrone was given by gavage together with 4 mg. of vitamin E on the 10th day of gestation. Similar injections of 1 mg. of progesterone and/or 0.5 μg. of estrone as well as the injections of 4 mg. progesterone and 10 mg. estrone did not produce any significant effect. Thus, the administration of progesterone or estrone at certain levels continuously or by a single dose either reduced or abolished the incidence of congenital malformations in maternal avitaminosis E.

PREVIOUS studies have shown that congenital malformations can be produced in the rat by maternal vitamin E deficiency (1, 2). Some preliminary observations on the histological and other changes in the term fetuses have been described (3, 4). Gross observations of the abnormal embryos from the 11th day of gestation onwards have recently been reported (5). The literature on the interrelationship between vitamin E and hormones was reviewed by Mason (6), who concluded that the preponderance of experimental evidence up to 1944 exonerated the anterior pituitary and thyroid as instigators of reproductive and other disturbances in vitamin E deficiency. However, recently Kaunitz (7) showed by ovarian transplantation experiments that there was some unspecific ovarian dysfunction in prolonged vitamin E deficiency. This fact was also borne

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out by the finding that the gonadotrophin content of the anterior pituitary of old, vitamin E-deficient male and female rats was significantly increased (8). Furthermore, after studying the effects of injection of alpha-estradiol, progesterone and tocopherol singly or in combination upon the uterine pigment formation of spayed, young vitamin E-deficient rats Kaunitz and associates (9, 10) suggested that progesterone and the tocopherols seemed to have functionally similar effects in preventing pigment formation in estradiol-injected, spayed females. Since the interrelationship between vitamin E and hormones is still in a state of controversy, it was deemed interesting to determine the effect of estrone and progesterone on the incidence of congenital abnormalities induced by maternal vitamin E deficiency.

EXPERIMENTAL METHODS

A total of 299 rats were used in the experiments reported here. The procedure used in the production of congenital abnormalities in maternal vitamin E deficiency has been reported previously (2, 5). Briefly, weanling female rats of Holtzman strain weighing approximately 40 gm. were put on a vitamin E-deficient ration consisting of 49% dextrin, 22% lard, 18% vitamin-free casein, 5% brewers' yeast, 4% salt mixture (U. S. No. XIV), and 2% cod liver oil. When they attained a weight of approximately 175 gm., they were mated with males which were fed stock pellets. After mating, as evidenced by the presence of sperm in the vaginal smear, they were assigned to different groups. Positive evidence of implantation was ascertained by observing the presence of erythrocytes in the vaginal smears during the second week of gestation. Two mg. of *d,l*-alpha-tocopherol acetate in 1 ml. of corn oil (Mazola) was given to each of the rats by stomach tube on the 10th day of gestation. This procedure has been used successfully in the production of abnormal young from vitamin E-deficient mothers. The rats with the supplementation of vitamin E only constituted the uninjected group. Seven groups of rats were injected daily subcutaneously with different levels of progesterone and estrone in 0.2 or 0.5 ml. of corn oil separately or in combination from the third day of gestation till term in addition to the vitamin E supplementation. Two groups of rats were injected daily with either 0.2 or 0.5 ml. of corn oil, the vehicle for the hormones, for the same duration to serve as controls. Also, positive controls which received 2 mg. of *d,l*-alpha-tocopherol acetate daily during the first 5 days of gestation, and negative controls which did not receive any vitamin E supplementation were used. All rats were laparotomized on the 21st day of gestation. The endocrine organs of many of these rats were weighed and fixed for histological examination, the results of which will be reported later.

For analysis of the statistical significance of the incidence of abnormalities and others the chi-square test was used. The significance of difference between groups with regard to numbers of implantation sites was assessed by means of the "Student" t test (11).

RESULTS

The results of the first series of experiments are presented in Table 1. The rats that had received hormone injections gained less weight during gestation than those without the injections, with those receiving 4 mg. of progesterone and 10 mg. of estrone gaining the least weight. Therefore, it appears that the administration of progesterone and estrone under the conditions of the present experiments did not improve the general health of the pregnant rats.

The percentage of normal fetuses in the uninjected group was reduced to 16.7% from the positive control of 90.3%. Further reduction of the percentage of normal fetuses occurred in rats receiving either 1 or 4 mg. of progesterone or 2 μg. of estrone. There were no normal live fetuses in rats receiving 0.5 μg. of estrone or various combinations of estrone and progesterone. The percentage of normal fetuses in the corn oil groups was about the same as that in the uninjected group. The negative control group receiving no tocopherol supplement yielded, as usual, no normal young.

TABLE 1. EFFECT OF PROGESTERONE AND ESTRONE ON THE INCIDENCE OF CONGENITAL ABNORMALITIES IN VITAMIN E-DEFICIENT RATS THAT HAD RECEIVED 2 MG. D,L-ALPHA TOCOPHEROL ACETATE ON THE 10TH DAY OF GESTATION

Treatment	Rats bred No.	Wt. gain during gestation, gm.	Live fetuses		Dead fetuses		Implantation sites[1]	
			Normal %[6]	Abnormal %[6]	Dead %[6]	Resorbed %[6]	Total No.	Average No.
Positive control[2]	46	73	90.3	0	0	9.7	420	9.1±0.9[8]
Uninjected	37	45[3]	16.7	5.3	1.0	77.0	280	7.8±2.4
Progesterone 1 mg.	14	28	2.5	0.8	0	96.7	119	8.5±2.7
Progesterone 4 mg.	19	35	9.3	0.9[7]	0.8	89.0	129	6.8±2.5[7]
Estrone 0.5 μg.	16	17	0	0	0	100.0	20	6.7[4]±2.0
Estrone 2 μg.	11	32	10.0	0[7]	0	90.0	68	6.1±2.2[7]
Progesterone 1 mg. +estrone 0.5 μg.	8	23	0	0	0	100.0	50	6.3±2.8
Progesterone 4 mg. +estrone 2 μg.	14	22	0	0[7]	0	100.0	76	5.4±2.5[8]
Progesterone 4 mg. +estrone 10 mg.	13	15	0	0	0	100.0	—	—
Corn oil 0.2 ml.	13	35	17.0	0[7]	1.0	82.0	82	6.3±2.4[7]
Corn oil 0.5 ml.	22	37	16.0	0[8]	1.0	83.0	179	8.0±2.8
Negative control[5]	40	26	0	0	0	100.0	359	9.0±1.9[7]

[1] Including those of live and non-resorbed dead fetuses.
[2] The rats in this group received 2 mg. daily of d,l-alpha-tocopherol acetate during the first 5 days of gestation.
[3] Average of 33 rats.
[4] Average of 3 litters.
[5] Vitamin E-deficient group. The rats did not receive any vitamin E supplementation.
[6] Calculated on the basis of total number of implantation sites, instead of on the number of live young obtained.
[7] $P < .05$ when compared with the uninjected group.
[8] $P < .01$ when compared with the uninjected group.

The most interesting result was a marked reduction in or abolition of the incidence of congenital abnormalities as one of the consequences of the hormone treatment. Calculated on the basis of total number of implantation sites, the incidence was 5.3% in the group without hormone treatment, but it was 0.8% and 0.9% in the groups with 1 and 4 mg. of daily progesterone injection, respectively. Also, no abnormal fetuses occurred in the groups given estrone treatment, singly or in combination with progesterone. The differences between the uninjected group and the group receiving 4 mg. progesterone or 2 μg. estrone, separately or in combination, were significant at the 5% level. Those between the uninjected and the corn oil control groups were also significant at the 1% and 5% levels, respectively.

The percentages of resportion were all increased in the groups injected either with the hormones or corn oil alone, with the groups receiving 0.5 μg. estrone and various combinations of progesterone and estrone showing complete resorption. The average number of implantation sites for the uninjected group was 7.8, which was significantly lower than in either the positive or negative control groups. However, the average number of implantation sites, as well as the percentage of abnormal young, was signifi-

cantly reduced when 4 mg. progesterone or 2 μg. estrone was injected singly or in combination during gestation.

Inasmuch as the continuous injection of either 0.2 ml. or 0.5 ml. of corn oil alone to the rats from the third day of gestation till term also abolished the appearance of abnormal young, the efficacy of the hormones in this respect might appear to be questionable. In order to pinpoint the effect of the hormones and to obviate the complication of the effect of continued injection of the vehicle, corn oil, another series of experiments was undertaken in which the rats were given by gavage a single dose of 4 mg. of progesterone or 2 μg. of estrone together with 4 mg. alpha-tocopherol

TABLE 2. EFFECT OF A SINGLE DOSE OF PROGESTERONE OR ESTRONE ON THE INCIDENCE OF CONGENITAL ABNORMALITIES IN THE OFFSPRING OF VITAMIN E-DEFICIENT RATS

Treatment on 10th day of gestation	Rats bred No.	Wt. gain during gestation, gm.	Live fetuses		Dead fetuses		Implantation sites[1]	
			Normal %[1]	Abnormal %[1]	Dead %[1]	Resorbed %[1]	Total No.	Average No.
Vitamin E 4 mg.	20	60	17.7	12.8	0.4	69.1	226	11.3±5.2
Vitamin E 4 mg. +progesterone 4 mg.	11	43	7.5[2]	0[2]	0	92.5[2]	80	8.9[3]±3.3
Vitamin E 4 mg. +estrone 2 μg.	15	56	26.2[2]	0[2]	0.8	73.0	130	8.7±2.1

[1] Calculated on the basis of total number of implantation sites.
[2] $P < .01$ when compared to groups with 2 or 4 mg. vitamin E alone.
[3] Average of 9 litters.

acetate dissolved in 1 ml. of corn oil on the tenth day of gestation. The use of 4 mg. of tocopherol was chosen in this series of experiments instead of the 2 mg. level since a higher incidence of abnormalities had been obtained with the 4 mg. level of supplementation (3).

The results are presented in Table 2. With a single oral dose of 4 mg. progesterone or 2 μg. estrone in conjunction with 4 mg. alpha-tocopherol acetate on the 10th day of gestation there was a complete abolition of the occurrence of congenital malformations in the live young produced while those rats receiving 4 mg. of tocopherol alone produced 12.8% abnormal fetuses. In the case of progesterone treatment there was a significant reduction in the percentage of the normal young with a concurrent increase in the percentage of resorption similar to those obtained in the first series of experiments. However, in the estrone experiment there was an increase in the percentage of normal young while the incidence of resorption remained about the same as in the control groups. There was no significant difference in the average number of implantation sites, nor in the maternal gain in weight during gestation, between the experimental and control groups.

DISCUSSION

As far as the percentage of abnormal young is concerned, it is apparent from the data in Table 1 that the injection of corn oil or ovarian hormones in corn oil from the 3rd day of gestation until term reduced the incidence of congenital abnormalities induced by feeding mother rats a vitamin E-deficient ration. It is not certain, from the data of this experiment, whether

the reduction of abnormal fetuses was due to the injection of corn oil or the hormones *per se*. It should be pointed out that in the case of corn oil injections, the abolition of abnormal fetuses occurred with corresponding increase in the percentage of resorption without affecting the percentage of normal young. The effects of corn oil in this respect could be due to either one of the following possibilities. Firstly, a change of the critical balance of the borderline deficiency of vitamin E, which is necessary for the production of congenital abnormalities, could have occurred. The presence of a trace amount of vitamin E (12) in corn oil might have changed the vitamin E content of the blood of the mother to such an extent that the delicate balance by which abnormality could occur was shifted slightly. Secondly, it is possible that the corn oil might contain an unknown factor which could prevent the occurrence of abnormal fetuses when used in quantities larger than 1 ml. Further investigation is required to elucidate this point.

The reduction or abolition of abnormalities in live fetuses by the injection of ovarian hormones in corn oil was not of the same nature as that brought about by corn oil alone as shown by the fact that in the former the percentage of resorption was greatly increased with slight to drastic reduction of the percentage of normal young. In the case of the combined injection of 0.5 μg. of estrone and 1 mg. of progesterone, or 2 μg. of estrone and 4 mg. of progesterone, or 10 mg. of estrone and 4 mg. of progesterone, complete resorption occurred, similar to the reproductive performance of female rats in the negative control group receiving no supplemental tocopherol. The physiological significance of these results are not entirely clear. It would appear that the continued injection of these hormones could inactivate the minute amount of tocopherol in the circulatory system resulting in total v itamin E deficiency.

Results from the second series of experiments where the hormones were administered by gavage at a level of 4 mg. of progesterone or 2 μg. of estrone together with 4 mg. of tocopherol in corn oil in a single dose on the 10th day of gestation offer more clear-cut effects. The use of 4 mg. of tocopherol was justified inasmuch as this dose tends to increase the incidence of abnormalities. It is quite apparent from data presented in Table 2 that the oral administration of either 4 mg. of progesterone or 2 μg. of estrone abolished the occurrence of the abnormal young. While both of these hormones abolished abnormalities, the mechanism of the abolition was probably of a different nature in each case. In the case of progesterone administration there was an increase in resorbed fetuses. Ershoff (13) noted that the daily injection of 150 I.U. of estrone, 4 mg. of progesterone or 2 mg. of lactogenic hormone to vitamin E-deficient rats from the 6th to the 16th day of pregnancy resulted in total resorption of fetal sites by the time of autopsy on the 16th day. As far as increasing the incidence of resorption is concerned it seems that the results obtained here are in accord with those of Ershoff although a physiological explanation is still not readily available.

In the case of estrone administration, the percentage of resorption was maintained at the level of the tocopherol treated group but the percentage of normal young was increased with total abolition of abnormal young. Thus, the oral administration of a single dose of 2 μg. of estrone on the 10th day appears to be effective in counteracting the teratogenic effect brought about by marginal vitamin E deficiency. It is quite possible that the teratogenic effect of this type of marginal nutritional deficiency was partially induced by an inadequate secretion of estrogens. Evidence on this point has been forwarded by Nelson, Lyon and Evans (14) who showed that estrogenic hormones afforded marked protection during pregnancy in pyridoxine-deficient rats.

Finally it might be interesting to point out the difference in the average number of implantation sites of the groups following the hormone administration between the first and second series of experiments. In the first series where the subcutaneous injection of the hormones was started on the third day post coitus, i.e., before implantation took place, there was a significant reduction in the average number of implantation sites in the groups receiving 4 mg. progesterone or 2 μg. estrone, separately or in combination. In the second series when the hormone administration took place on the 10th day when implantation had occurred, there was no significant reduction in the average number of implantation sites. Our results from estrogen experiments confirm the previous observations of Courrier and Gros (15) in the cat. These authors found that if estrogen is given in the interval between insemination and implantation it prevents the implantation and neutralizes the progestational changes in the uterus. However, after implantation, gestation is very difficult to interrupt with estrogen.

REFERENCES

1. Thomas, B. H. and D. W. Cheng: *Proc. Iowa Acad. Sci.* **59:** 218. 1952.
2. Cheng, D. W. and B. H. Thomas: *Proc. Iowa Acad. Sci.* **60:** 290. 1953.
3. Cheng, D. W.: *Iowa State Coll. J. Sci.* **30:** 340. 1954.
4. Cheng, D. W. and B. H. Thomas: *Anat. Rec.* **121:** 274. 1955.
5. Cheng, D. W., L. F. Chang and T. A. Bairnson: *Anat. Rec.* **129:** 167. 1957.
6. Mason, K. E.: *Vitamins and Hormones* **2:** 107. 1944.
7. Kaunitz, H.: *3rd Int. Cong. on Vit. E., Venice*, p. 35. 1955.
8. Pan, S. Y., H. B. Van Dyke, H. Kaunitz and C. A. Slanetz: *Proc. Soc. Exper. Biol. & Med.* **72:** 523. 1949.
9. Kaunitz, H., C. A. Slanetz and W. B. Atkinson: *Proc. Soc. Exper. Biol. & Med.* **70:** 302. 1949.
10. Atkinson, W. B., H. Kaunitz and C. A. Slanetz: *Ann. N.Y. Acad. Sci.* **52:** 68. 1949.
11. Snedecor, G. W., *Statistical Methods*, Iowa State Coll. Press. 1946.
12. Quaife, M. L.: *J. Biol. Chem.* **175:** 605. 1948.
13. Ershoff, B. H.: *Anat. Rec.* **87:** 297. 1943.
14. Nelson, M. M., W. R. Lyons and H. M. Evans: *Endocrinology* **52:** 585. 1953.
15. Courrier, R. and G. Gros: *Compt. rend. soc. biol.* **120:** 5 and 8. 1935.

COMPARATIVE EFFECTIVENESS OF ESTROGENS ON FLUID IMBIBITION AND GROWTH OF THE RAT'S UTERUS[1]

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ABSTRACT

Five natural estrogens (estradiol-17B, estrone, estriol, equilin and equilenin) and one synthetic estrogen (diethylstilbestrol) were compared as to effectiveness in promoting fluid imbibition and growth of the uterus in 22-day old rats. Graded amounts of each estrogen were injected subcutaneously as a single dose in 0.1 cc. sesame oil and uterine responses observed at intervals up to 72 hours. Both quantitative and qualitative differences were found with respect to dose required for a response and speed, duration, and extent of the reaction. Some estrogens are more effective for fluid imbibition and others more active for growth and superiority for one does not imply superiority for the other.

It was found that the minimal dose in micrograms required to produce 33⅓% increase in uterine wet weight at six hours (Astwood unit) is for estradiol, 0.025; estriol, 0.029; diethylstilbestrol, 0.078; equilin, 0.312; estrone, 0.45; and equilenin, 0.546. The dosage required to produce 70% gain in uterine wet weight at six hours is estriol, 0.078; estradiol, 0.1; diethylstilbestrol, 0.156; equilenin, 0.625; estrone and equilin, 1.25. The dose at which maximal imbibition occurs at four hours rather than at six hours is estriol, 0.039; estradiol, 0.15; diethylstilbestrol, 0.625; equilin, 5; and equilenin and estrone, 50.0. The order of potency for uterine growth between the thirtieth and fortieth hours by the dose required for maximal fluid imbibition on the sixth hour was found to be diethylstilbestrol, 0.1; estradiol, 0.1; equilin, 1.25; estriol, 5.0; estrone, 1.25; and equilenin, 1.25. While the order of effectiveness for growth at large doses, up to 50 μg., was diethylstilbestrol, estradiol, equilin, estrone, equilenin and estriol.

ESTROGENIC compounds, both natural and synthetic, vary widely in their capacity to promote growth of the different structures of the female reproductive tract. There is also a marked species difference in responsiveness. Of two animals most commonly used, the mouse is much more sensitive than the rat. However, comparisons of activity are usually based upon a single reaction in a particular test animal, and the response most frequently selected in the past has been vaginal cornification. The chief advantage of this method is its simplicity and the fact that the same animals can be used repeatedly, but the data obtained are in large measure qualitative and subjective in that they depend upon the evaluation of cellular contents of a vaginal lavage. There is also a disadvantage in that

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it is difficult to excise tissues accurately from the vaginal wall for biochemical studies. A structure best suited for comparative studies of the physiology of estrogens should be one that is not only responsive, but also discrete and easily accessible. The uterus comes most nearly meeting these requirements.

Astwood (1) studied the sequence of changes in fluid content and weight of the uteri of 21 to 23-day old rats following single injections of graded doses of estradiol. This procedure not only made it possible to evaluate the response in terms of dosage but also related the succession of events with respect to time. He found that the response to a given dose of estradiol followed a characteristic pattern. The first noticeable effect was hyperaemia associated with accumulation of fluid in the tissues and uterine lumen. This phase of the response was maximal at six hours, followed by a decrease in weight due to a partial resorption of fluid between the ninth and twelfth hours. At this point the uterus entered into a growth phase which attained a maximal extent by the thirtieth hour.

The time-relationship of uterine responses to estrogen has been used by others in more recent research. Notable among these are the observations by Telfer (2) on the influence of estradiol on nucleic acids, respiratory enzymes and the distribution of nitrogen in the rat uterus, and the experiments by Mueller (3) on the incorporation of radio-active amino acids in uterine tissue under the influence of estrogen. This problem has been approached in the present study by comparing the individual capacities of five natural estrogens (estradiol-17B, estrone, equilin, and equilenin) and one synthetic compound (diethylstilbestrol) to induce fluid imbibition and growth of the uterus.

METHODS

The animals used in the present investigation were normal sexually immature 22-day old female rats of the Harvard strain weighing 40 to 50 grams. The estrogens were furnished in crystalline form by courtesy of the manufacturer,[2] and a stock solution of each was prepared by serial dilution. The estrogens at all dosage levels were administered subcutaneously in 0.1 cc. of sesame oil and the animals were killed at selected times following a single injection.

The animals were killed by cervical dislocation, the uteri were immediately dissected out and freed from the adhering tissue, weighed on a torsion balance and placed in a tared vessel for dry weight determinations. For dry weights, the tissues were placed in an oven at a temperature of 100 to 115 degrees centigrade and weighed twenty-four hours later on a microbalance. Nitrogen determinations were made at the beginning of the study but these values so closely paralleled absolute dry weights that this was not included as a routine procedure.

In those instances where expressed uterine weights were desired, the uteri were removed as above, weighed, and then nicked with scissors, the luminal contents gently pressed out on moist paper toweling and reweighed. All uterine weights, unless other-

[2] We are very grateful to Park, Davis and Company, Schering Corporation and Ayerst Laboratories for generous gifts of natural estrogens.

wise stated, are for the tissue plus the luminal contents. The wet weights of uteri that were distended with luminal fluid, are slightly less than true values, as in the process of removal it is impossible to prevent the loss of a small portion of the fluid when the uterine cervix is cut.

A four-hour and six-hour dose response curve was determined for each estrogen for the study of fluid imbibition. Two dosage levels were used in evaluating the relative growth promoting properties of the different estrogens. A high dosage of fifty micrograms and a low dosage, determined by using the minimal amount of hormone giving a maximal fluid imbibition within six hours.

Approximately eight to ten animals were used to establish the position of each point on a particular curve. The data on uterine wet and dry weights and percentage of water were analyzed statistically to determine the degree of uniformity within each group by using the formula derived for use with calculating machines (8).

$$\text{Standard error} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{\frac{n-1}{n}}}.$$

RESULTS

Fluid imbibition

Fluid imbibition increases proportionally with the dose of estrogen administered and the following is noted when a graded series of doses is given and the uteri weighed six hours later: a) The percentage differences in weight gained are greatest at low dosages, and decrease as the tissue reaches its maximal ability to respond, b) Amounts of an estrogen greater than that required for a maximal response at six hours not only do not produce a further gain in weight but may reach a point at which the response is less than that for a smaller dose (Figs. 1–6).

The drop in uterine weight occurring on the sixth hour at high dosages of estrogens would appear to be due to a self-inhibitory effect, but such is not the case. Uterine weights obtained at the end of four hours for high dosages may be higher than those at six hours for the same dose (Figs. 1–6). Also, when the four-hour responses are examined it is again noted that in many instances, as in the six-hour test, there is a falling off of uterine weights with higher dosages. The same explanation may again be applied, as in certain instances three-hour responses were found to be higher than those for the corresponding four- or six-hour test (Fig. 11). Thus, it can be stated: as the dosage of an estrogen is increased the time of maximal fluid imbibition occurs earlier than that obtained with smaller dosages.

All gains in weight during the first six hours are due mainly to the accumulation of fluid in the lumen of the uterus. Dry weights for these uteri show an increase over those of the control but this is misleading. The gain is due to the solid material present in luminal fluid, as shown by the fact that when luminal fluid is expressed the uterine dry weight is essentially the same as that for the untreated control.

The percentage of total water parallels uterine wet weight. As uterine

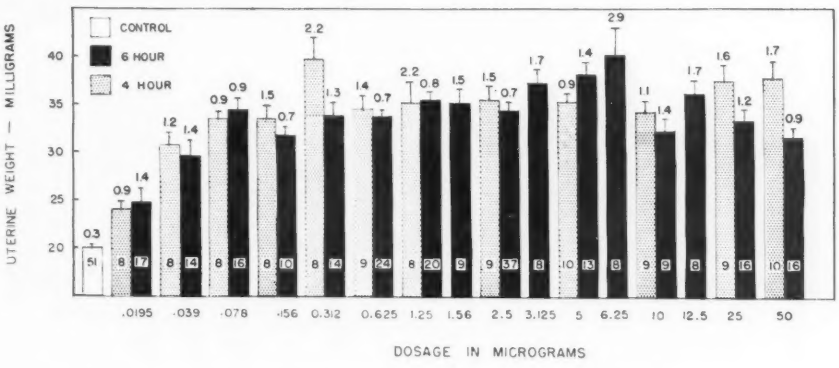

FIG. 1. Four and six hour response to estriol. The standard errors are shown at the top of the bars in figures 1 to 6, and the number of animals used is indicated on the base of each bar.

weight increases with successive dosages so does the percentage of water. Likewise, when the amount of water is decreasing, during the period of resorption, uterine wet weights also decrease.

Dry weights show a different stituation when the six and nine hour responses to a low dosage are compared with their percentages of water. The percentage of water differs by three percent in the total amounts while the absolute dry-weights are the same. A possible explanation is, that solid material is carried into the lumen as a part of the luminal fluid, but during resorption mostly water is removed, resulting in a fluid of greater concentration.

Estrogens differ in their ability to promote fluid imbibition. To facilitate comparisons, estradiol was used as the standard. An Astwood unit is that

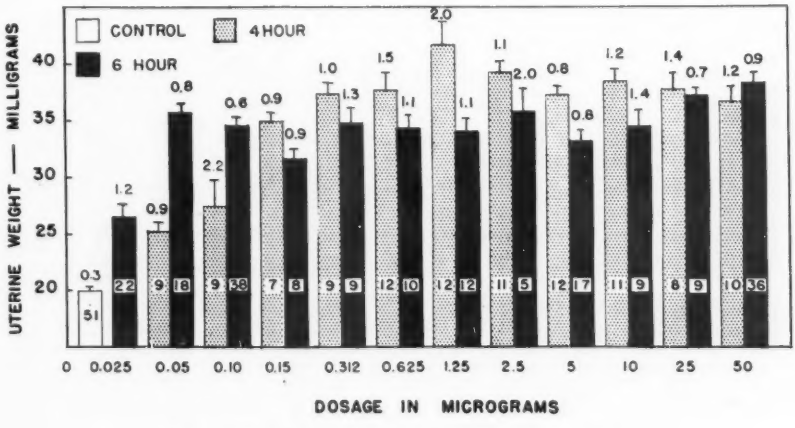

FIG. 2. Four and six hour response to estradiol.

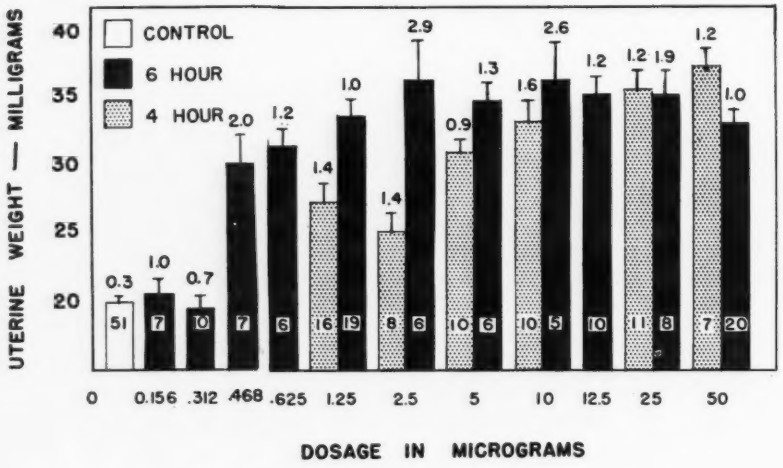

FIG. 3. Four and six hour response to estrone.

amount of estrogen which will elicit an increase in uterine wet weight of $33\frac{1}{3}\%$ in six hours. This unit is equal to 0.025 μg. of estradiol. The order of estrogenic potency from strongest to weakest, in micrograms equivalent to one Astwood unit, is as follows: estradiol, 0.025; estriol, 0.029; diethylstilbestrol, 0.078; equilin, 0.312; estrone, 0.45; and equilenin, 0.546. Estradiol and estriol are almost equal and they in turn are about three times as strong as diethylstilbestrol, $12\frac{1}{2}$ times stronger than equilin, 18 times as strong as estrone, and 21.8 times stronger than equilenin in producing uterine fluid imbibition.

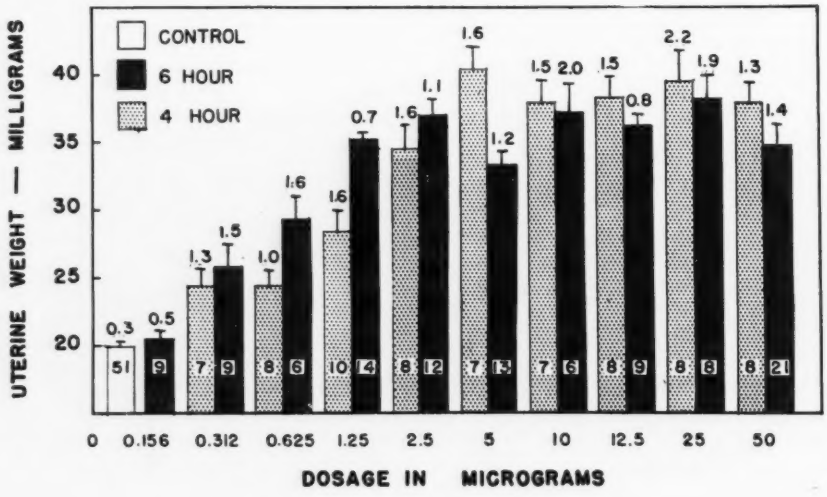

FIG. 4. Four and six hour response to equilin.

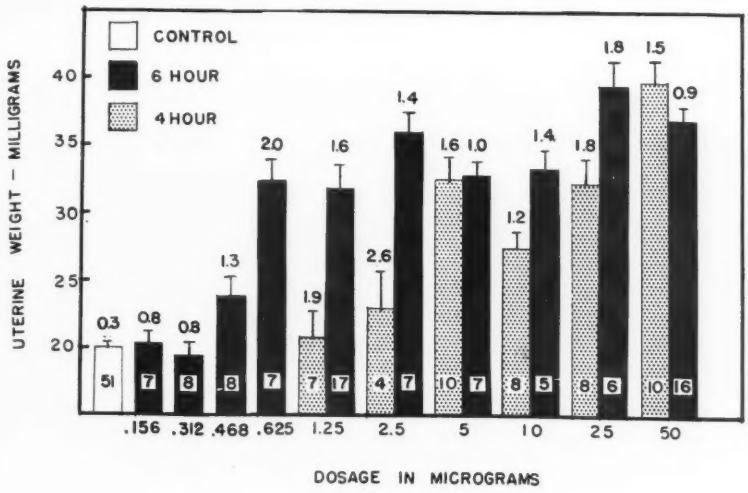

FIG. 5. Four and six hour response to equilenin.

The minimal dosage of estradiol producing a maximal response in six hours is 0.05 microgram. The uterine weight so obtained represents an increase of 75% over the control. The microgram amounts of the various estrogens required to produce a 70% increase, in their order of activity, are: estradiol, 0.05; estriol, 0.078, diethylstilbestrol, 0.156; equilenin, 0.625; estrone and equilin, 1.25. These results show that estradiol is $1\frac{1}{2}$ times stronger than estriol, 3 times stronger than diethylstilbestrol, $12\frac{1}{2}$ times stronger than equilenin, and 25 times more potent than estrone or equilin.

The dosage of an estrogen required to produce uterine weights at four

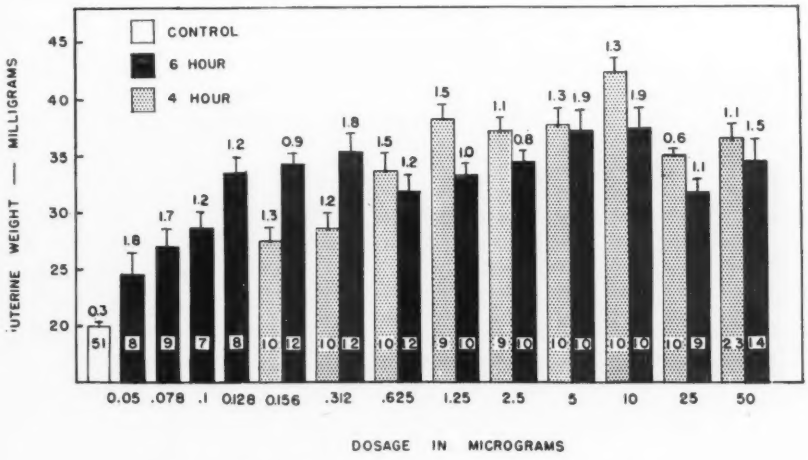

FIG. 6. Four and six hour response to diethylstilbestrol.

hours that are greater than those at six hours seems as characteristic for each compound as the amount required for an Astwood unit. A possible exception is estriol for which the four-hour response was first the greater at 0.039 μg. followed by considerable irregularity between the two responses as the dosage was increased (Fig. 1). With this possible exception, the minimal amounts of the different estrogens required to bring this about when listed in the order of strength in micrograms are as follows: estriol, 0.039; estradiol, 0.15; diethylstilbestrol, 0.625; equilin, 5; and equilenin and estrone, 50. It is seen that for this reaction estradiol is probably about one fourth as strong as estriol, four times stronger than diethylstilbestrol, 32 times stronger than equilin, and 320 times more potent than either equilenin or estrone.

Another comparison that can be made from the data obtained for these six estrogens is the greatest increase in uterine weight that each can induce in six hours regardless of dosage within the range used. When compared on this basis it is seen (Figs. 1–6) that the maximal weight attained by uteri on estradiol in this time was 35 mg. at a dosage of 2.5 μg. Approximately the same maximal weights were obtained for estrone at 2.5 μg. and diethylstilbestrol at 5 μg., while equilin at 10 μg. produced uteri somewhat larger. Estriol at 6.25 μg. and equilenin at 25 μg. are capable of producing uteri weighing more than forty milligrams which is at least a hundred per cent increase over the controls.

These observations indicate that if the early response to an estrogen is properly evaluated it is necessary to compare the dose-response curves for both four and six hours. This is well illustrated by estriol which at 50 μg. gives the same response as that for 0.156 μg. on the sixth hour. Examination of the four- and six-hour responses to corresponding intermediate doses of estriol shows they are quite different. The response to 0.156 μg. falls on the rising leg of the curve of fluid imbibition while that for 50 μg. is in the phase of water resorption having already reached a maximum at four hours. These effects as described for estriol are generally true for the other five estrogens although diethylstilbestrol seems to act more slowly in that a maximal response to a low dosage is reached at nine hours instead of six.

These observations show that fluid imbibition is not only a response common to all estrogens but that they differ widely in their ability to produce the effect. Also, the response to a particular estrogen is constant and characteristically related to time and dosage. On the basis of these differences, it can be stated that estradiol and estriol are approximately equal with respect to microgram amounts required to produce an Astwood unit, but three times more diethylstilbestrol, 12½ times more equilin, 18 times more estrone, and 21.8 times more equilenin is required to produce the same effect. However, for the production of 70% increase in uterine weights, estradiol is the most efficient, followed by estriol, diethylstilbestrol

equilenin, estrone and equilin in that order. The data for the five estrogens also show that as the dosage is increased maximal imbibition occurs earlier, and that they differ with respect to the amount necessary to bring this about.

Uterine growth

Stimulation of uterine growth is a general property of estrogenic compounds, but some are more effective than others. Rapid growth begins between the ninth and twentieth hour in a typical response to a single injection of an estrogen, after which the rate progressively decreases as the uterus approaches its maximal response. Maximal uterine weights are usually attained by the fortieth hour followed by cessation of growth and involution, which begin at about the forty-eighth hour and are complete by the seventy-second hour at which time weights of the uteri approach those of untreated controls (Figs. 7–12).

The percentage of water in general reflects the physiological state of the uterus. During the first few hours fluid imbibition greatly increases and then falls rapidly during resorption, though it never reaches the control level, and again rises with the initiation of growth. When growth begins, as measured gravimetrically, water increases proportionate to uterine weight but never equals that of the first four or six hours during rapid fluid imbibition. Two exceptions to this general statement are the responses to equilin and diethylstilbestrol which at high doses continue the formation of luminal fluid in the presence of growth (Figs. 10 and 12).

Uterine dry weight is a reflection of uterine wet weight, as it too increases proportionately during growth. This is not due to solid material present in luminal fluid, but to tissue growth, as the dry weights of uteri from which the luminal fluid has been expressed are much greater than those of the controls. It is probably true that in the intact uterus during the growth phase a small portion of the dry weight may be due to solids of luminal fluid that have not been resorbed and certainly so when concurrent growth and imbibition occur such as that following injections of large doses of diethylstilbestrol or equilin. However, in general the uterine dry weights increase as growth progresses and decrease during the subsequent tissue involution.

Both high and low dosages of the estrogens were used for comparisons of growth responses following a single injection. The high dosage was in each instance fifty micrograms while the minimal amount of each compound required to give maximal water imbibition in six hours was used for the low dosage. The larger amount was selected arbitrarily while the smaller doses were based on a biological reaction and consequently probably should be considered as being more nearly physiological. However, the high dosages brought out certain growth characteristics that were not evident when small doses were given, for example, some estrogens were

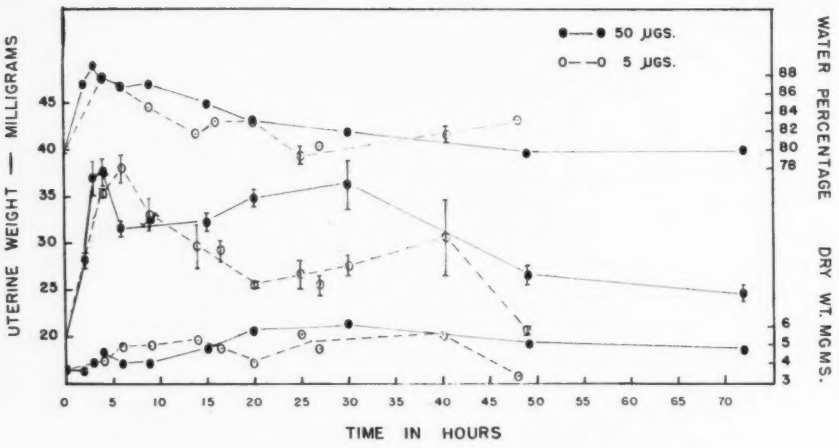

FIG. 7. Growth response with estriol. In figures 7 to 12 the vertical lines represent the standard error.

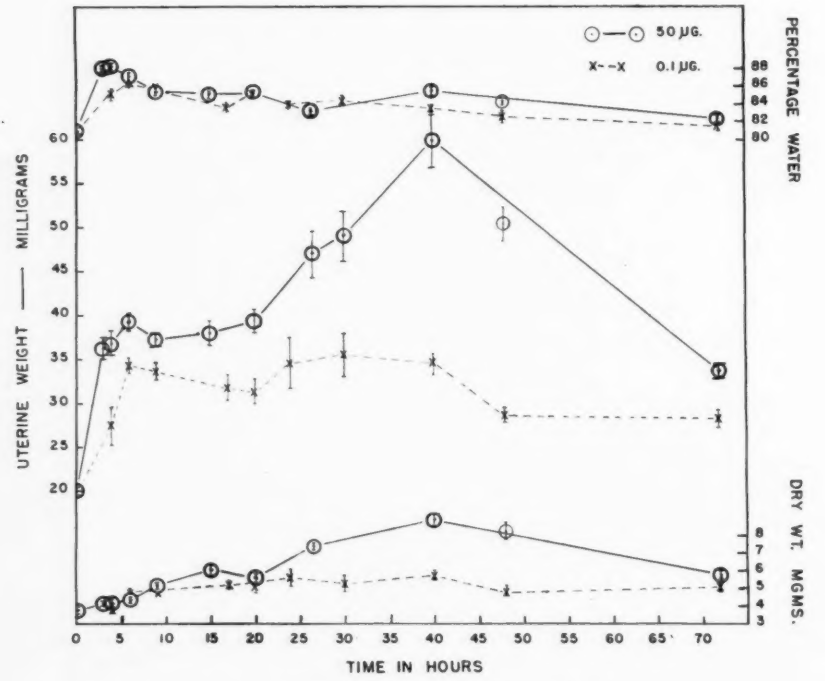

FIG. 8. Growth response with estradiol.

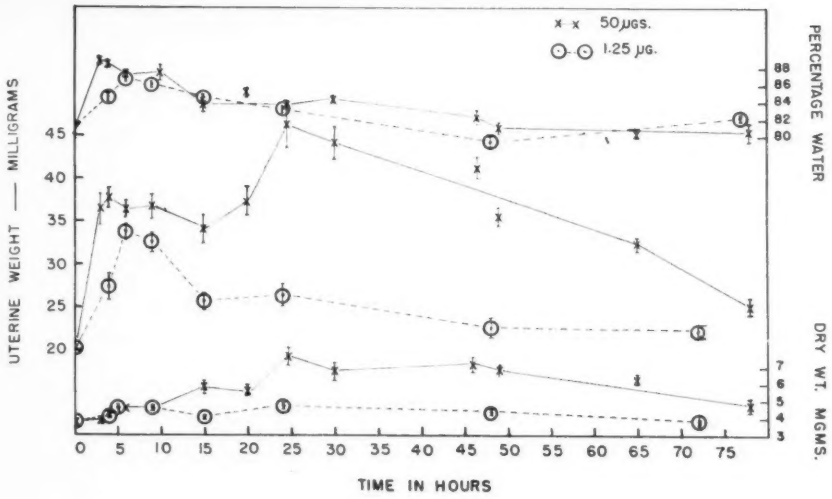

FIG. 9. Growth with estrone.

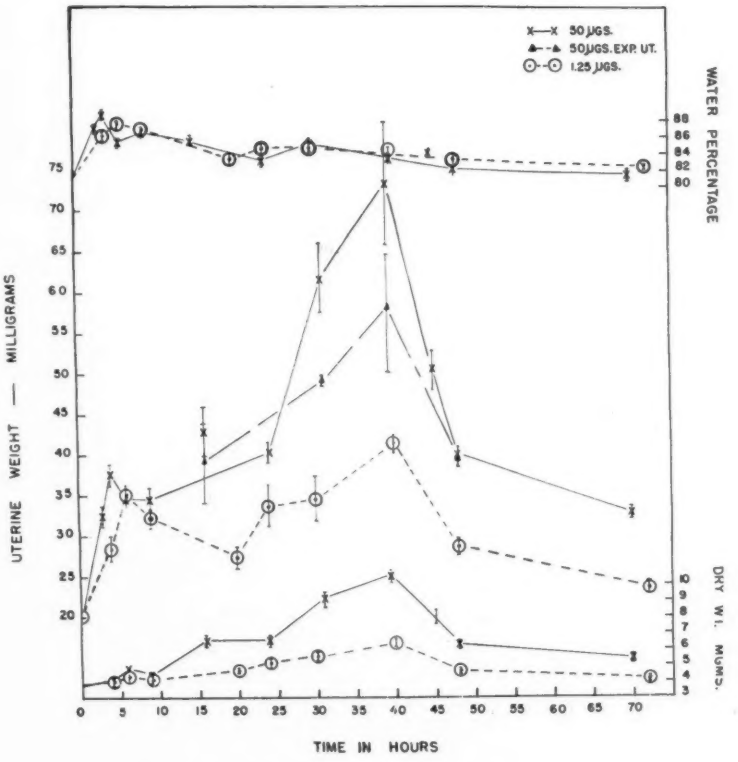

FIG. 10. Growth response with equilin.

found to be more active over longer periods of time than others. Also, the maximal growth with 50 μg. was greater for long acting estrogens, while those that acted for a shorter time produced their maximal effect earlier in the growth phase and the subsequent decline was more rapid (Figs. 7–12). These differences were due probably in part to difference in solubility of the steroids but this does not seem to offer a complete explanation.

Comparisons of growth produced by a dose of each estrogen based on the amount required to induce maximal fluid imbibition in six hours showed that these two effects are quite independent responses (Figs. 7–12).

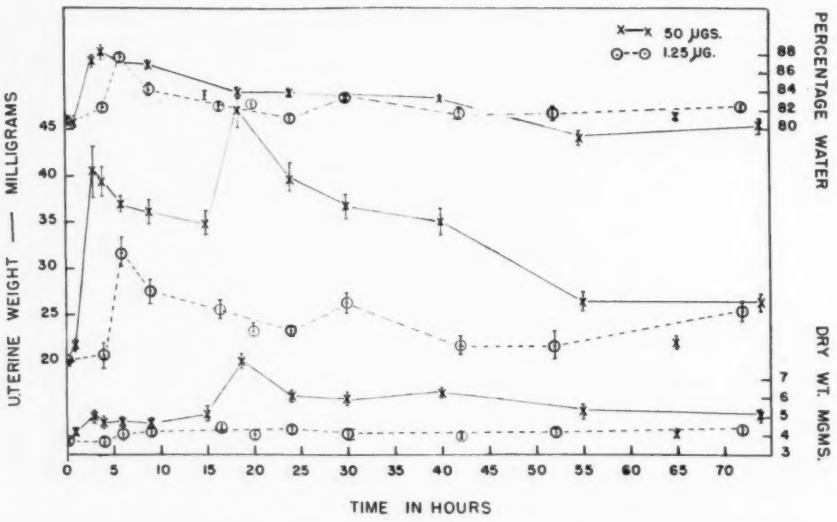

FIG. 11. Growth response with equilenin.

Some that are strong promoters of fluid imbibition are relatively weak for growth (equilenin and estriol) while others (estradiol and equilin) are equally effective for both. Equilenin and estriol in doses of 1.25 μg. give a good response for fluid imbibition at six hours (Figs. 1 and 5) and slight growth at 30 to 40 hours, while even 5 μg. estriol gives a poor growth response (Figs. 7 and 11).

The curves representing growth induced with fifty micrograms of the different estrogens (Figs. 7–12) show certain shifts in position with respect to each other during the growth phase. While the synthetic estrogen, diethylstilbestrol, is the strongest growth promoter and estriol is the weakest, the other four, estradiol, equilin, estrone, and equilenin, change their order of activity. Their relative positions at twenty hours in order of greatest activity are equilenin, estrone, estradiol, and equilin. However, when the respective growth curves are compared it is noted that equilenin,

which ranks first at twenty hours, has already induced even greater uterine weights earlier, and its effect is in fact subsiding while at twenty-four hours equilenin has dropped to fifth place, being replaced by estrone. Equilin, at thirty and forty hours, is followed by estradiol, estrone (now sub-

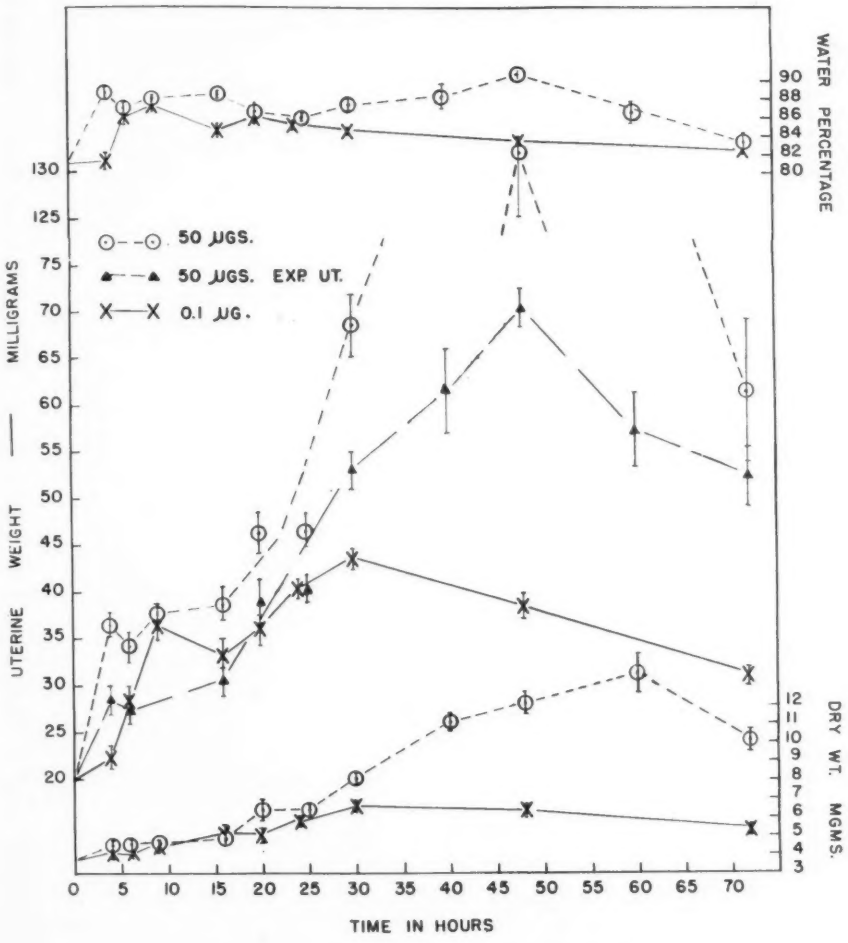

FIG. 12. Growth response with diethylstilbestrol.

siding), and equilenin. At forty-eight hours estradiol has replaced equilin, (now subsiding) otherwise the order remains the same as at forty hours. Diethylstilbestrol and equilin promote both uterine growth and fluid imbibition simultaneously, but even so such uteri after the fluid has been expressed, have wet weights at thirty and forty hours equal to those of animals given estradiol. The relative effectiveness of the six estrogens in

order of their ability to stimulate over-all growth would appear to be diethylstilbestrol, estradiol, equilin, estrone, equilenin, and estriol, among which equilenin and estrone seem to act more rapidly than the others and consequently produce their effects at an earlier time.

These studies tend to show that while the action of estrogens are in general the same, each has its own individual peculiarities both as to dosage required for a particular reaction and the character and extent of the response. Although certain differences in potency and duration of action have been mentioned by many investigators these usually have been accredited to variation in rate of absorption, method of administration, rate of metabolic destruction and other factors rather than to the possibility of a physiological individuality of the compounds.

DISCUSSION

Many investigators have tested the relative activities of estrogens by various standardization procedures and their observations on uterine growth are in general agreement with our own. Also Szego and Roberts (9) found that in rats 24 to 27 days old the relative effectiveness of estrogens in promoting the uptake of water by the uterus was of the order estriol, estradiol-17B and estrone. However, a more detailed analysis of the action of estrogens on fluid imbibition and uterine growth has brought out more clearly certain individual diffffferences.

It is obvious that certain variables need to be taken into consideration in evaluating such differences. Among these are variations in rate of absorption, rate of metabolic destruction, and methods of administration. In the present study all estrogens were given subcutaneously in 0.1 ml. sesame oil. There was little difference in character of the response in these experiments and others in which 0.1 ml. of propylene glycol was used. Szego and Roberts (9) found that imbibition of uterine fluid in young rats on the sixth hour was in general the same when estrogens were given intraperitoneally either in 0.2 ml. oil or as sodium salts in physiological saline. Apparently the most important difference was a three-fold increase in the response to estriol when given in saline while estradiol and estrone were equally effective in both. The order of effectiveness remained the same, being estriol, estradiol, and estrone which is in agreement with our observations. We have found that the estrogens when listed in order of their ability to produce approximately 70% increase in uterine wet weight by the sixth hour are estriol, estradiol, diethylstilbestrol, equilenin, estrone and equilin. Though much is yet unknown regarding the nature of estrogenic action, our data indicate that estrogens may differ both as to the amount required for a particular reaction and as to the character of the response. Some are more effective for imbibition of uterine fluid while others are more active in the promotion of uterine growth and superior capacity for one does not necessarily indicate superior capacity for the

other. For example, estradiol is the strongest of the natural estrogens for growth and excels at low doses for fluid imbibition while equilin ranks second to estradiol at all doses for growth, is weak at low doses for imbibition, but is very effective for both growth and imbibition at high doses.

As to the physiological meaning of these differences, nothing definite is known but it may be significant that ordinarily two or more estrogens are present and presumably act concurrently in the promotion of a response. Estradiol-17B and estrone are apparently hormones of the estrous cycle, while in women estradiol-17B and estrone and a small amount of estriol are present during the menstrual cycle (4), and in pregnancy, estriol becomes by far the dominant hormone (5, 6). A similar situation seems to hold for the mare. Estradiol-17B and estrone are found during the estrous cycle while in pregnancy at least three additional steroids appear; equilin, equilenin and dihydro-equilenin, of which the first two are the most abundant (7). The observations in the present study on two estrogen effects, fluid imbibition and growth, suggest the possibility that under normal conditions the combined effect of the several estrogens may be one in which some are more effective than others for certain aspects of the reaction or that the action of one may have been limited by that of another (10, 11). Also, the abundance of estriol in women and equilin and equilenin in mares during pregnancy strongly suggests that these steroids are hormones of pregnancy and the differences in activity reported in this study seem to support the thought that they may indicate adaptations for the performance of physiological processes peculiar to gestation.

REFERENCES

1. Astwood, E. B.: *Endocrinology* **23**: 25. 1938.
2. Telfer, M. A.: *Arch. Biochem. and Biophys.* **44**: 111. 1953.
3. Mueller, G. C.: *J. Biol. Chem.* **204**: 77. 1953.
4. Engel, L. L., R. Wilson, W. R. Slaunwhite, Jr., P. Carter, P. A. Olmsted, and I. T. Nathanson: Ciba Foundation Conf. Colloquia on Endocrinology, **2**: 104. J. and A. Churchill, Ltd., London. 1952.
5. Doisy, E. A.: *Endocrinology* **30**: 933. 1942.
6. Diczfalusy, E.: Acta Endocrinologica, supplementum 12, part 2, pp. 85–155. 1953.
7. Dorfman, R. I. and F. Unger: Metabolism of steroid hormones. Burgess Publishing Co. 1953.
8. Snedecor, G. W.: *Statistical Methods.* Fourth edition, p. 91. The Iowa State College Press. 1946.
9. Szego, C. M. and S. Roberts: *Recent Progress in Hormone Research*, **8**: 419. 1953.
10. Hisaw, F. L., J. T. Velardo and C. M. Goolsby: *J. Clin. Endocrinol and Metab.* **14**: 1134. 1954.
11. Huggins, C. and E. V. Jensen: *J. Exp. Med.* **102**: 335. 1955.

NOTES AND COMMENTS

DEPRESSION OF THE MOUSE THYROID IODIDE PUMP BY GOITROGENS

ABSTRACT

The administration of thiouracil or propylthiouracil to mice maintained on a moderately low iodide diet results in depression of the activity of the iodide pump per unit of thyroid weight (expressed as the thyroid/serum radioiodide gradient, T/S. The activity of the pump per gland (T/S) increases with increase in total thyroid weight, increase in T/S or increase in both.

Several groups (1, 2, 3) have reported that thyroid iodide pump activity (T/S) of rats is increased after the administration of thiouracil (TU) or propylthiouracil (PTU). Lipner *et al.* (4) and Scow and Greer (5) have observed that the administration of PTU to mice decreases the T/S. This report presents the data on which the abstract of Lipner *et al.* (4) was based.

METHODS

Thirty-five C_3H-HeN male mice three months old were randomized into seven groups and placed on an adequate stock diet of natural foodstuffs moderately low in iodine.[1] To this diet were added varying amounts of either PTU or TU (Table 1), and the animals were fed their respective diets until the experiment was terminated at the end of four weeks.

TABLE 1. DEPRESSION OF THE THYROID IODIDE PUMP BY GOITROGENS

Treatment	Mouse wt., gm.	Thyroid weight	T/S	t/S	Mean acinar cell height
Control	24.4 ± 0.8	1.70 ± 0.12*(4)†	197.3 ± 11.8	3.35	9.5 ± 0.3
1250 μg. TU‡	23.8 ± 0.3	16.72 ± 2.07 (5)	119.6 ± 2.5	20.0	14.9 ± 0.4
2500 μg. TU	20.4 ± 0.8	18.60 ± 2.0 (4)	146.6 ± 11.0	27.3	13.7 ± 0.3
5000 μg. TU	22.9 ± 1.0	11.96 ± 1.08 (5)	191.1 ± 15.6	22.9	14.6 ± 0.3
125 μg. PTU§	23.0 ± 1.6	13.88 ± 2.06 (5)	119.6 ± 13.2	16.5	17.5 ± 0.4
250 μg. PTU	23.9 ± 0.8	17.46 ± 2.24 (5)	132.8 ± 6.5	23.2	16.9 ± 0.3
500 μg. PTU	23.4 ± 0.6	15.30 ± 2.26 (5)	168.9 ± 9.3	25.1	16.8 ± 0.2

* Standard Error of the Mean = $\frac{\text{range}}{\text{No. of Animals}}$ (8)

† Number of animals.
‡ Thiouracil/gm. diet.
§ Propylthiouracil/gm. diet.

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[1] The composition of the Morris moderately low iodide diet No. 77 is as follows:

Skim milk	22.75%
Ground whole wheat	61.25%
Dehydrated brewer's yeast	4.00%
NaCl	1.40%
Ferric citrate	0.13%
Cod liver oil	0.50%
Corn oil	9.70%
	100.00%

A modified version of this diet No. 77-A in which the cod-liver oil was replaced by a vitamin A and D concentration in corn oil contained by analysis 0.75 ± 0.039 μg. of iodine/gm. of diet (6).

The T/S was determined as described by Halmi *et al.* (3). Forty-five minutes after the acute blocking dose of 1.0 mg. PTU per mouse, the mice received 1 μc of I^{131} injected subcutaneously on the back in the region over the pelvis. The animals were killed between 60 and 90 minutes after the I^{131} was administered. The planchet technic of VanderLaan and Greer (2) for determining the I^{131} content of thyroids and serum was used. The radioactivity was determined by means of a Geiger-Muller counter. The T/S was calculated from the ratio of radioiodide per 100 mg. thyroid tissue to the radioiodide per 0.1 ml. serum. The t/S was calculated from the ratio of radioiodide per total gland to the radioiodide per 0.1 ml. of serum (6) or t/S = T/S × total gland weight/100.

The thyroids were dissected under a binocular microscope at 9× magnification and weighed immediately on a Roller-Smith torsion balance. Radioactivity was determined on one lobe. The other lobe was fixed in SUSA, embedded in paraffin, and sectioned at 5μ for histological examination. One cell was selected for cell height measurement from each acinus that appeared to be sectioned through the center. The sections were projected on a screen and in each section the acinar height of 20 cells was measured.

RESULTS AND DISCUSSION

The data in Table 1 indicate that PTU and TU had an effect on mouse thyroid weight and acinar cell height similar to that seen in the rat. The effect of these drugs on mouse thyroid function when the T/S is used as the measure was dissimilar. Compared with the controls both PTU and TU depressed the T/S at low dose levels. At the highest dose level TU was less effective than the lower doses in stimulating thyroid growth and there was no depression of T/S. PTU caused a progressive increase in T/S as the dose increased.

Halmi (7) has suggested that the t/S is a more reliable index of iodide pump activity than the T/S especially when gland weights are greatly different. The t/S calculation applied to the data of Scow and Greer (5) indicates that the iodide pump activity per gland increases with increased gland weight (7). Table 1 shows the t/S and T/S for the present data. In the mouse the mechanism responsible for iodide accumulation does not increase at the same rate that thyroid weight increases. As a result T/S decreases while t/S rises. From the point of view of the whole animal, the t/S is a more meaningful index of thyroid function than is the T/S which reflects the functional status of a unit quantity of the gland.

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REFERENCES

1. VanderLaan, J. E. and W. P. VanderLaan: *Endocrinology* **40**: 403. 1947.
2. VanderLaan, W. P. and M. A. Greer: *Endocrinology* **47**: 36. 1950.
3. Halmi, N. S., B. N. Spirtos, E. M. Bogdanove and H. J. Lipner: *Endocrinolgy* **52**: 19. 1953.
4. Lipner, H. J., B. P. Wagner and H. P. Morris: *Fed. Proc.* **13**: 465. 1954.
5. Scow, R. O. and M. A. Greer: *Endocrinology* **56**: 590. 1955.
6. Lipner, H. J., B. P. Wagner and H. P. Morris: *Proc. Soc. Exp. Biol. and Med.* **87**: 578. 1954.
7. Halmi, N. S.: *Ciba Foundation Colloquia on Endocrinology* **10**: 79. 1957.
8. Mantel, N.: *Amer. Statistician* **5**: 26. 1951.

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LIMB REGENERATION IN THE SALAMANDER, *TRITURUS VIRIDESCENS*, AFTER LARGE INITIAL AND DURING PROLONGED SMALLER INJECTIONS OF CORTISONE ACETATE[1]

ABSTRACT

A study of the effect of cortisone on limb regeneration was made with the salamander, *Triturus viridescens*. One group of 50 animals was given a large initial dose of 4000 μg of cortisone acetate at the time of amputation of the left foreleg. A second group of 50 animals was given small doses of cortisone acetate over a period 3 to 4 weeks at the rate of 1000 μg per injection every two days until a total dosage of 12,000 μg was attained.

Blastema formation was delayed from 2 to 6 days after which regeneration was normal in comparison to the controls. Neither epithelium nor connective tissue growth were inhibited after the formation of the blastema.

The inhibition of growth of connective tissue in general by cortisone has been demonstrated by numerous investigators, and its selectivity for mesenchymal tissue acknowledged. The study of wound healing following mechanical injury to the skin in cortisone treated laboratory animals was the method of investigation most frequently used (1, 2, 3). Local thinning of the epidermis and dermal connective tissue results following applications of cortisone to areas of the rat neck (4, 5, 6). Parenteral administration of adrenocorticotropin also causes thinning of the dermal connective tissue (7).

This study was undertaken to determine the effect of cortisone on limb regeneration in the salamander. The fact that this investigation is concerned with a cold-blooded vertebrate should not be ignored. It is not to be assumed that results obtained by research on mammals can necessarily be extrapolated directly to the cold-blooded vertebrates.

MATERIAL AND METHODS

Adult *Triturus viridescens* maintained in the laboratory in a specially designed aquarium (8), were employed in this study. The experimental animals were divided into two groups of 50 adult animals each. In addition there were 25 control animals in each experiment.

The first group were given a large initial dose of cortisone acetate at the time of amputation of the left foreleg. The amount of the dose was 4 mg. (4000 μg.) in a saline suspension. Twenty-five controls were given only the saline base.

The second group received a total dosage of 12 mg. (12,000 μg.) of cortisone acetate over a period of 3 to 4 weeks after amputation of the left foreleg. One mg. was given every two days. The 25 controls received only the saline base in the same amount and at the same time as the experimentals. All injections were given intraperitoneally.

The regenerates studied histologically were fixed in 10% formalin and serially sectioned at 8 microns. The sections were stained with Mallory's stain.

RESULTS

In the first group four animals died a few days after the injection. About 75% of the remaining 46 animals showed a retardation in the formation of the blastema as compared to the controls. This retardation varied from 3 to 6 days. After 4 to 6 weeks no differences could be detected between the size of the regenerates of the experimental and control animals.

Although there was a delay of 3–6 days in blastema formation of experimentals in comparison to the controls, regeneration proceeded normally, and the resulting regenerates were equal in size to those of the controls. Histological studies did not reveal any suppression of connective tissue growth.

Several experimental animals in the second group showed an early necrosis of the stump, but all were normal in a few weeks. About 50% of the experimental animals showed a delay of about 2 to 3 days in formation of the blastema. No difference could be detected after 6 weeks in the regenerating capacities of the experimental animals in

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[1] Supplied through the courtesy of Merck & Co., Inc.

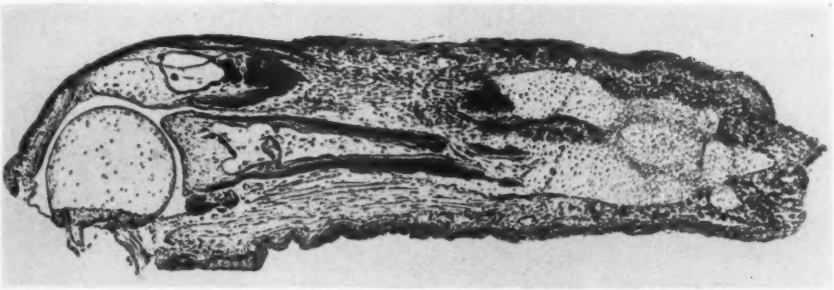
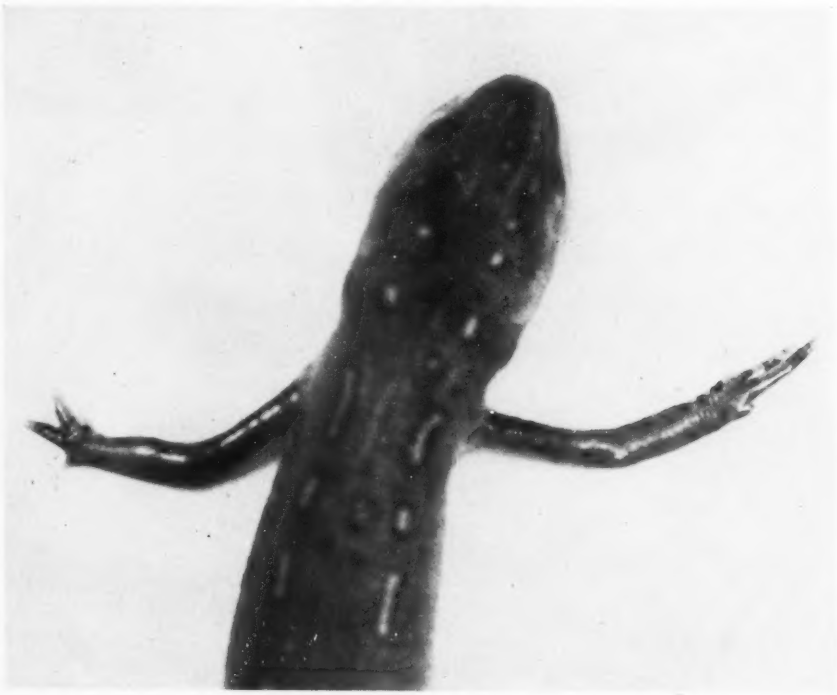


FIG. 1

comparison with the controls. Cortisone failed to suppress the growth of connective or epithelial tissues as was the case in the first experiment.

DISCUSSION

The results of this study indicate that cortisone does not inhibit growth of amphibian tissue as depicted by regeneration in the salamander. Comparable results were obtained by Manner (9) with a small daily injection of cortisone acetate (0.005 mg.). His study was concentrated on the early stages of regeneration, and reported a delay in blastema formation of about 4 days.



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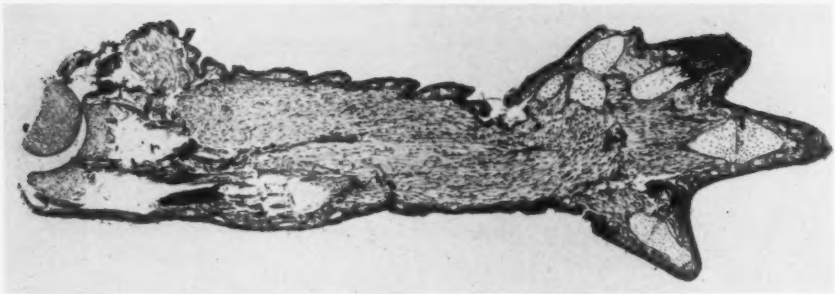

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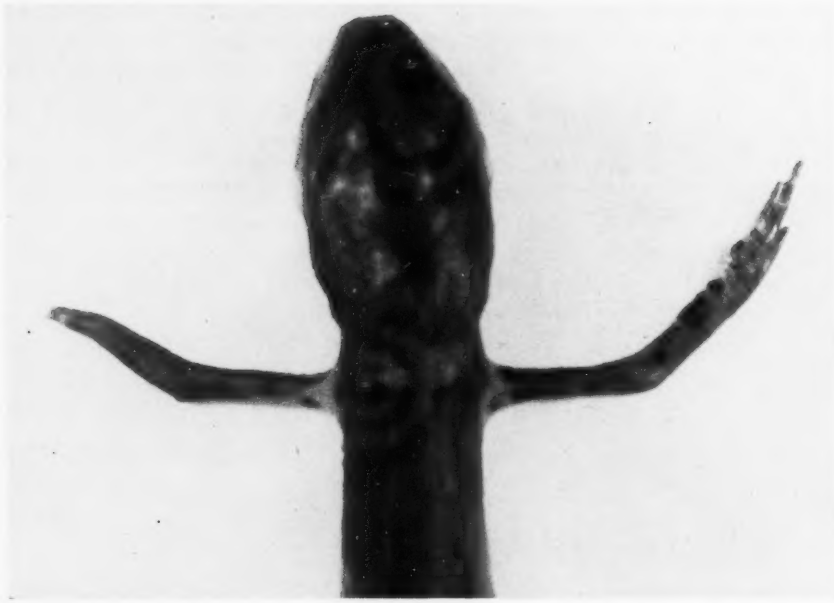

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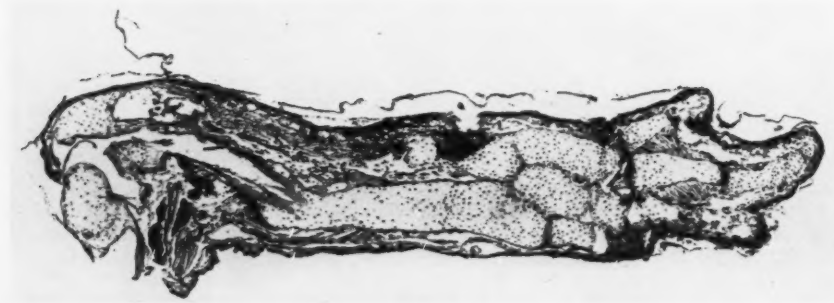

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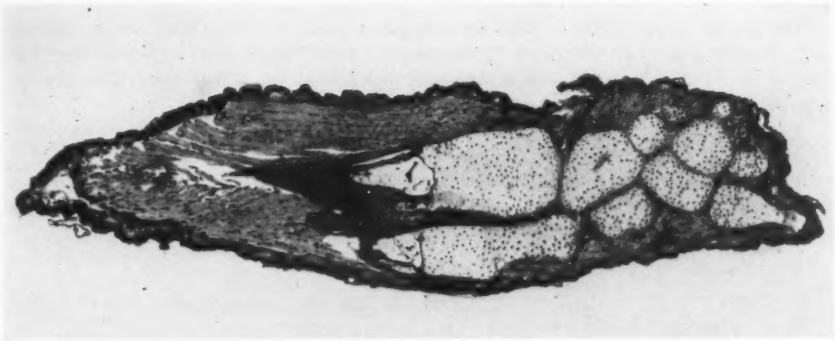

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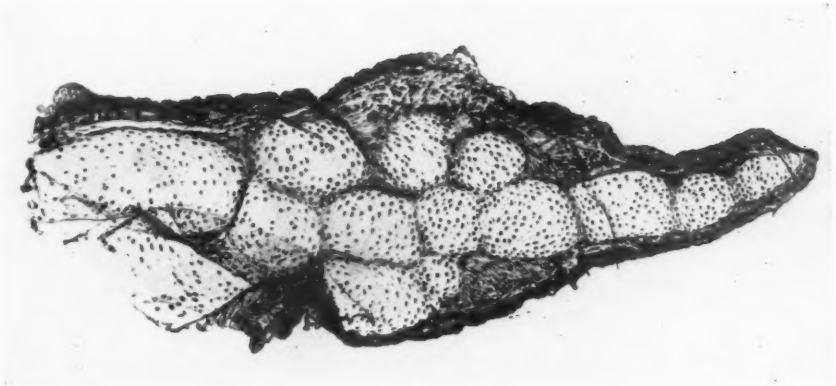

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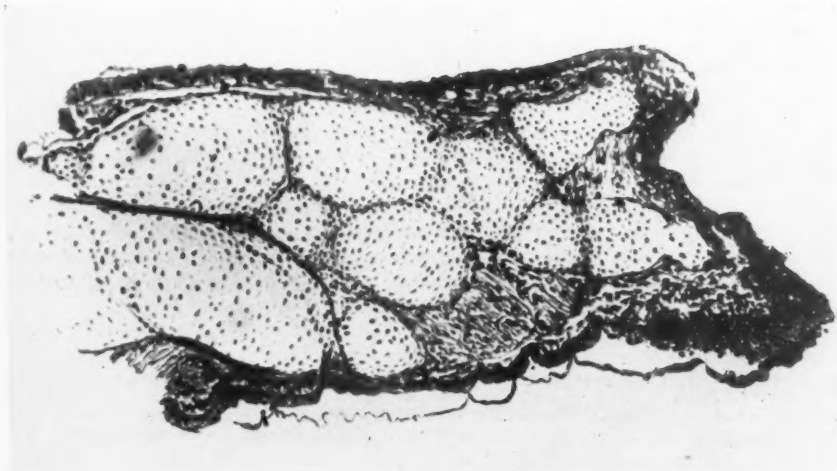

FIG. 8

The present study indicates that the salamander will tolerate a high dosage of cortisone without untoward effects on its regenerative capacities. There is, however, a thinning of the epidermis in the wound area (9) which does not persist after blastema formation, even though injections may be continued beyond that point.

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REFERENCES

1. RAGAN, C., E. L. HOWES, C. M. PLOTZ, K. MEYER AND J. W. BLUNT: *Proc. Soc. Exper. Biol. and Med.* **72**: 718. 1949.
2. RAGAN, C., E. L. HOWES, C. M. PLOTZ, K. MEYER, J. W. BLUNT AND R. LATTES: *Bull. N. Y. Acad. Med.* **26**: 251. 1950.
3. LENTI, G., *et al: Folia Endocrinology* (*Pisa*) **4**: 743. 1956.
4. BAKER, B. L. AND W. L. WHITAKER: *Anat. Rec.* **102**: 333. 1949.
5. BAKER, B. L.: *Endocrinology* **46**: 544. 1950.
6. BAKER, B. L. AND C. W. CASTOR: *Anat. Rec.* **106**: 173. 1950.
7. BAKER, B. L., D. J. INGLE, C. H. LI AND H. M. EVANS: *Anat. Rec.* **102**: 313. 1948.
8. WILLIAMS, D. D.: *Nature* (*London*) **181**: 933. 1958.
9. MANNER, H. W.: *Growth* **19**: 169. 1955.

THE INFLUENCE OF GELATIN IN THE ASSAY OF PURIFIED PARATHYROID EXTRACTS

ABSTRACT

Parathyroid extracts purified by solvent distribution appeared to have lost activity when assayed in calcium-depleted, parathyroidectomized rats. The addition of gelatin as a vehicle to these extracts almost completely restored their original potency in a 4-hour test; there was little effect at 8, and none at 24 hours. When gelatin was added to Parathyroid Extract, U.S.P. (Lilly), no increase in potency was observed.

The potentiating effect of the vehicle with purified preparations might be explained by delayed absorption from the injection site.

During studies on the fractionation of parathyroid extracts it was found that activity seemed to be lost during the course of purification. Previous observations with purified corticotropin (1) suggested that rate of absorption might be a factor and that the addition of gelatin as a vehicle might increase the apparent potency of the purer materials. Moreover, Rasmussen and Westall, using a different assay method, reported a shortened duration of action of a partially purified preparation (2). They further found that the activity of their material was prolonged by suspension in arachis oil. This report concerns the influence of gelatin as a vehicle in the assay of partially purified parathyroid hormone.

MATERIALS AND METHODS

The extract used in the experiments reported here was prepared by butanol extraction of Parathyroid Extract U.S.P. (Lilly) dissolved in 1% NaCl-6% acetic acid (3). After adding one-half volume of water the butanol was removed under reduced pressure at 60° C in a rotatory evaporator. The activity was then precipitated from aqueous solution with 2%

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TABLE 1

	Parathyroid extract, U.S.P.	Parathyroid extract, U.S.P. plus gelatin
Group	A	G
Number animals	15	15
Average log dose (12.5 U.S.P. units)	1.0969	1.0969
Average response Serum Ca (mg./100 ml.)	10.27	10.07
Slope	3.1157	2.8895
Combined slope		3.0026
Potency of G compared to A		86% (50–210% at the 95% significance level)

trichloroacetic acid. Following conversion to the acetate form with an ion-exchange resin, the material was diluted with weak acetic acid to the appropriate concentration for assay. Gelatin, when used, was added to the assay dilutions as an equal volume of a 30% solution. All fractions were administered subcutaneously at equal final concentration of activity in 0.2 to 0.8 ml. volumes. A modification of the bioassay of Munson (4) was used: eighty-gram male rats were fed a calcium-deficient diet for four days and then parathyroidectomized and injected immediately with the assay material. The small variation of body weight of the rats was neglected. Samples of sera obtained four hours after parathyroidectomy were assayed for calcium using alizarin (5). Parathyroid Extract, U.S.P., was used as the bioassay standard in all cases. Results of assays were analyzed according to Irwin (6).

RESULTS

A three-dose comparative assay of Parathyroid Extract, U.S.P., in gelatin solution against the same material without gelatin showed no significant difference in potency (Table 1). However, the vehicle greatly enhanced the effect of the material prepared by butanol extraction. Figure 1 represents the results of a two-dose assay comparing this partially purified substance with and without gelatin against the standard in gelatin. The diminished responses and slope produced by the gelatin-free preparation fell below the assay range. Thus, these results could not be compared on a bioassay basis, but a "t" test showed the difference in response between the purified preparation alone and

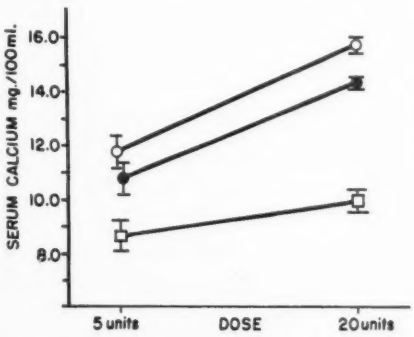

FIG. 1. Two-dose assay comparing partially purified parathyroid extract alone □—□, and in gelatin ●—●, vs. Parathyroid Extract, U.S.P., in gelatin ○—○. Each point represents the mean value (±S. E.) obtained from 5 animals.

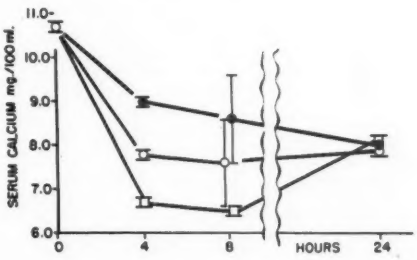

FIG. 2. Concentration of calcium in sera of rats parathyroidectomized at time zero and injected immediately with partially purified extract alone ○—○, or with gelatin added ●—●. The points represent the mean value (±S. E.) using 5 or 6 rats per group. The control group □—□, was parathyroidectomized, but not injected.

the purified preparation in gelatin to be highly significant ($p = <.05$ for the 5 unit dose; $p = <.001$ for the 20 unit dose).

The influence of gelatin as a vehicle on the ability of this preparation to prevent the fall in concentration of calcium in the serum of the assay animals is shown in Figure 2. Each injected rat received the equivalent (approximately 0.2 mg. dry weight) of 20 units of the starting material. The animals were allowed a normal diet after eight hours in an attempt to prevent death from hypocalcemia. Nevertheless, two of the five control animals died before eight hours, and a third before twenty-four hours following parathyroidectomy. Addition of the vehicle to the extract caused a significant difference in the concentration of calcium in the sera four hours following parathyroidectomy and injection ($p = <.05$), but at eight hours and beyond the differences were not significant.

DISCUSSION

The addition of gelatin as a vehicle almost completely restored the apparent loss of activity of the material studied here. A similar effect has also been found when assaying fractions prepared by paper chromatography. These observations, in addition to those of Rasmussen and Westall, suggest that admixture with appropriate vehicles is necessary in following the course of purification of this hormone.

The mechanism through which gelatin potentiated the action of partially purified extracts was not elucidated. Perhaps the enhanced effect was due to slower release of the hormone from subcutaneous tissues. Such an explanation is supported by Munson's conclusion that the hormone has a short life *in vivo* (4). The large amount of inert protein in Parathyroid Extract, U.S.P., may act in a similar manner. Thus, when gelatin was added to that material, no increase in potency was observed. However, prevention of inactivation in dilute solutions, or prevention of adsorption to glass, might have contributed to the observed effect.

Preparations of higher purity isolated by countercurrent distribution sustained greater losses of activity which were not completely restored by the adjuvant (3). The cause of this further apparent inactivation was not found.

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REFERENCES

1. Raben, M. S., I. N. Rosenberg, V. W. Westermeyer and E. B. Astwood: *J. Am. Med. Assoc.* **148:** 844. 1952.
2. Rasmussen, H. and R. G. Westall: *Nature* **178:** 1173. 1956.
3. Aurbach, G. D., R. N. Beck and E. B. Astwood: *Fed. Proc.* **17:** 7. 1958.
4. Munson, P. L.: *Ann. N. Y. Acad. Sciences* **60:** 776. 1955.
5. Natelson, S. and R. Penniall: *Anal. Chem.* **27:** 434. 1955.
6. Irwin, J. O.: *Suppl. J. Roy. Stat. Soc.* **4:** No. 1, 1937.

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PREVENTION OF ADRENAL-REGENERATION HYPERTENSION BY AN ALDOSTERONE-BLOCKER

ABSTRACT

Chronic treatment of rats with the aldosterone-blocker 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone prevented adrenal-regeneration hypertension and the accompanying cardiac hypertrophy. Unaffected were: mortality, body weight, saline intake, renal and adrenal hypertrophy.

A steroidal lactone was recently described (1) that was found to antagonize the renal excretory effects of mineralocorticoids in laboratory animals (2, 3) and man (4). This compound was also found to have a hypotensive action in metacorticoid hypertensive rats and to prevent the pressor action of desoxycorticosterone acetate in saline-fed rats (5, 6). It was therefore of interest to determine the ability of the steroid to prevent the induction of adrenal-regeneration hypertension in rats, a disease that may be due to hyperreactivity to aldosterone (7). This condition can be prevented or reversed by adrenal depression with Amphenone (8).

MATERIAL AND METHODS

Twenty-four, female, Sprague-Dawley rats, 26 days old, were subjected to a one-stage right adrenalectomy, right nephrectomy, and left adrenal enucleation, following Skelton's procedure (9). They were offered Rockland Rat Diet and 0.86% sodium chloride solution *ad libitum* and, 2 days post-operatively, half of the rats received subcutaneous injections of 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (SC-5233) and the other half received equivalent volumes of the propylene glycol solvent. SC-5233 dosage was 20 mg./kg. twice a day on weekdays and 40 mg./kg. once a day on weekends for 8 weeks. Once a week, blood pressures (10), body weights, and fluid intakes were measured; the whole experiment was conducted in a rigidly-controlled "blind" fashion (11). Statistical procedures were based on the rank-sum test (12); standard errors are listed only to indicate degree of variability.

RESULTS AND DISCUSSION

Chronic treatment with SC-5233 prevented the rise in blood pressure and the cardiac hypertrophy without appreciably affecting mortality, body weight, spontaneous saline intake, or renal and adrenal hypertrophy (Tables 1, 2). It is tempting to postulate that

TABLE 1

	Weeks of treatment							
SC-5233 group	1	2	3	4	5	6	7	8
No. rats	12	12	12	12	11	11	10	9
Blood press., mm.	148 ± 2	142 ± 2*	152 ± 2†	159 ± 1†	159 ± 1§	160 ± 2†	153 ± 3	153 ± 2§
Body wt., gm.	106 ± 3	133 ± 2	160 ± 2	179 ± 4	182 ± 7	183 ± 7	213 ± 11	217 ± 10
NaCl intake, ml./rat/day	45 ± 3	62 ± 4	67 ± 4	64 ± 4	64 ± 4	60 ± 7	70 ± 6	59 ± 5†
Placebo group								
No. rats	12	12	12	12	12	11	10	9
Blood press., mm.	152 ± 2	149 ± 2	161 ± 2	165 ± 2	171 ± 2	175 ± 1	166 ± 2	170 ± 1
Body wt., gm.	107 ± 3	136 ± 2	161 ± 2	174 ± 6	174 ± 8	185 ± 12	209 ± 5	208 ± 5
NaCl intake, ml./rat/day	54 ± 6	68 ± 6	75 ± 7	65 ± 6	70 ± 9	85 ± 6	86 ± 6	82 ± 5

Means ± standard errors.
* $P < 0.02$, † $P < 0.01$, § $P < 0.001$.

TABLE 2

Group	Organ weights ± S.E., mg./100 gm.			Mortality
	Adrenal	Kidney	Heart	
SC-5233	20 ± 2	740 ± 27*	274 ± 11†	3/12
Placebo	20 ± 2	843 ± 50	378 ± 22	3/12

* $P = 0.13$, † $P < 0.001$.

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SC-5233 acted by interfering competitively with the mineralocorticoids secreted by the regenerating adrenal. However, it is quite possible that the blood pressure effects of the compound are independent of its blocking effect on the renal excretory actions of mineralocorticoids. This was suggested by the observations that the 19-nor derivative of SC-5233, though a more potent blocker at the kidney level (2, 3), lacked the hypotensive action of SC-5233 in metacorticoid hypertension (5, 6), a disease that is initiated by corticoid-sodium overdosage but that soon becomes self-sustaining. With other renal blockers as well, including steroids with and without the lactone configuration, there has been a general lack of correlation between hypotensive action and the ability to block the electrolyte excretory patterns produced by mineralocoticoids. Therefore, it is not possible at this time to suggest a mechanism for the effect of SC-5233 on adrenal regeneration hypertension.

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REFERENCES

1. CELLA, J. A. AND C. M. KAGAWA: *J. Am. Chem. Soc.* **79:** 4808. 1957.
2. KAGAWA, C. M., J. A. CELLA AND C. G. VAN ARMAN: *Science* **126:** 1015. 1957.
3. KAGAWA, C. M., F. M. STURTEVANT AND C. G. VAN ARMAN: *J. Pharm. & Exper. Therap.* In press, 1958.
4. LIDDLE, G. W.: *Science* **126:** 1016. 1957.
5. STURTEVANT, F. M.: *Feder. Proc.* **17:** 413. 1958.
6. STURTEVANT, F. M.: *Science* **127:** 1393. 1958.
7. MASSON, G. M. C., S. B. KORITZ AND F. G. PERON: *Endocrinology* **62:** 229. 1958.
8. CHAPPEL, C. I., J. CAHILL, G. RONA AND G. A. GRANT: *Endocrinology* **62:** 30. 1958.
9. SKELTON, F. R.: *Proc. Soc. Exper. Biol. & Med.* **90:** 336. 1955.
10. KERSTEN, H., W. BROSENE, F. ABLONDI AND Y. SUBBAROW: *J. Lab. & Clin. Med.* **32:** 1090. 1947.
11. STURTEVANT, F. M.: *J. Pharm. & Exper. Therap.* **121:** 369. 1957.
12. WILCOXON, F.: *Biom. Bull.* **1:** 80. 1945.

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LIPID DEPLETION OF THE ZONA GLOMERULOSA OF THE RAT ADRENAL CORTEX AFTER ADMINISTRATION OF 9-ALPHA-FLUOROHYDROCORTISONE[1]

ABSTRACT

Nine-alpha-fluorohydrocortisone is physiologically ambivalent, acting as a potent glucocorticoid and mineralocorticoid. Administration of 0.25 mg. of this material per 100 gm. of body weight, subcutaneously, 3 times weekly, for 7 weeks to young and old, female, Sprague-Dawley rats caused selective lipid depletion of the

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zona glomerulosa. This lipid-depleting effect of 9-alpha-fluorohydrocortisone was practically identical to changes induced in rats given desoxycorticosterone acetate. However, the younger rats required the "conditioning" effect of exogenous ACTH given with the 9-alpha-fluorohydrocortisone before the glomerulosa lipid-depleting effect could take place. These results are interpreted to mean that, in the rat, the mineralocorticoid activity of 9-alpha-fluorohydrocortisone is more manifest on the zona glomerulosa of the adrenal cortex, than is its glucocorticoid effect. The results also suggest that ACTH may have some conditioning effect on the responsiveness of the secretory activity of the zona glomerulosa in the rat.

The zona glomerulosa of the adrenal cortex secretes mineralocorticoids, e.g., aldosterone, desoxycorticosterone (1). High levels of desoxycorticosterone-like steroids (2), changes in sodium levels (3) and hypertensive vascular disease (4) have been associated with changes in the width or lipid content of this zone. Changes in the zona fasciculata have been correlated with circulating levels of the glucocorticoids (5). The morphological and functional implications of these cortical zones have been reviewed by Greep and Deane (5). Aldosterone excretion is augmented in congestive heart failure (6), eclampsia (7), nephritis and nephrosis (8), ascites (9) and hypertension (10). For this reason it would be important to learn more concerning the specific functional roles of the zones of the adrenal cortex, what controls them, and how they respond to exogenous and endogenous stimuli.

A recently prepared synthetic analogue of hydrocortisone, 9-alpha-fluorohydrocortisone has been demonstrated to possess a most unusual type of activity, i.e., it is a potent glucocorticoid (11–13) and at the same time, equal to or more potent than aldosterone as a mineralocorticoid (14–16). While studying this compound we encountered some unusual effects on certain endocrine organs. The most striking effect was on the zona glomerulosa of the adrenal which is reported here.

MATERIALS AND METHODS

This work is taken from a larger study, to be reported later, in which 21-day-old and 12 month-old female, Sprague-Dawley rats were unilaterally nephrectomized, and treated with a variety of steroids. Regular rat chow (Rockland) was fed and no saline was added to the drinking water. Nine-alpha-fluorohydrocortisone[2] (in saline) was given as 0.25 mg./100 gm. of body weight (b.w.), subcutaneously (s.c.), three times weekly to both the young and old rats. Similarly, cortisone (2.5 mg./100 gm. b.w., s.c.) and desoxycorticosterone acetate (DCA) (2.5 mg./100 gm. b.w., s.c.) were given separately to animals on a similar regimen. Additional groups of young and old rats were given the above dose of 9-alpha-fluorohydrocortisone in conjunction with $\frac{1}{2}$ unit of ACTH (gel)/100 gm. of b.w., s.c., three times weekly. After 7 weeks of treatment the animals (12 animals per group) were sacrificed. Adrenal glands were fixed in 10% neutral formalin, sectioned on a freezing microtome at 10 μ, and stained for lipid content with Sudan orange (hematoxylin counterstain) and Sudan black B (Meyer's carmalum counterstain).

RESULTS

The very potent glucocorticoid effects of 9-alpha-fluorohydrocortisone were demonstrated by the severe involution of the thymus and spleen in both young and old animals. However, loss in body weight and adrenal atrophy were not marked. Mineralocorticoid effects were manifested by the development of severe hypertension, kidney damage, and increased heart size. Severe arteriosclerosis was found in the aortae of the older rats (to be reported). This may, or may not be a manifestation of mineralocorticoid activity. Concomitant with the above changes there was complete and selective depletion of the lipid material in the zona glomerulosa (Fig. 3) in all of the older animals receiving this hormone. The lipid depletion of the zona glomerulosa produced by 9-alpha-fluorohydrocortisone resembled similar depletion in rats given DCA. However, an interesting age factor appeared to be operative in the zona glomerulosa response to

[2] We are indebted to Dr. Henry A. Strade of the E. R. Squibb Company for a gift of 9-alpha-fluorohydrocortisone.

FIG. 1 Adrenal cortex of non-treated, young, female weanling, Sprague-Dawley rat. All zones are filled with lipid. (Sudan black B, 10 μ, $\times$50.)

FIG. 2. Complete, selective depletion of the zona glomerulosa of a young, female weanling given 0.25 mg. of 9-alpha-fluorohydrocortisone and $\frac{1}{3}$ unit of ACTH (gel) per 100 gm. body weight, subcutaneously, once daily, every other day, for 7 weeks. (Sudan black B, 10 μ, $\times$50.)

FIG. 3. Lipid depletion of the zona glomerulosa of a year-old, female Sprague-Dawley rat given 9-alpha-fluorohydrocortisone only (same dose as in Fig. 2). Note greater hypertrophy of cortex in older animal. (Sudan black B, 10 μ, $\times$50.)

9-alpha-fluorohydrocortisone. All animals, young and old, given DCA showed lipid depletion of the zona glomerulosa. None of the younger animals given 9-alpha-fluorohydrocortisone alone exhibited any evidence of lipid depletion of the zona glomerulosa. If the younger animals were given ACTH in conjunction with the 9-alpha-fluorohydrocortisone they consistently showed very marked lipid depletion of the zona glomerulosa comparable to that seen in the older animals (Fig. 2). In no case (total of 264 animals) was this selective lipid-depleting effect on the zona glomerulosa observed except in those cases mentioned above.

DISCUSSION

The disappearance of lipid from one of the zones of the adrenal cortex usually connotes decreased biosynthesis by that zone. In these experiments DCA and 9-alpha-fluorohydrocortisone both caused selective depletion of the zona glomerulosa. Cortisone produced disuse atrophy and lipid depletion of the zona fasciculata. It is particularly interesting that 9-alpha-fluorohydrocortisone which is "ambivalent" physiologically (11–16) showed no histological effects on the zona fasciculata but very dynamic lipid-depleting effect on the zona glomerulosa; this, in spite of the fact that it had marked glucocorticoid activity in these same animals, i.e., severe involution of the thymus and spleen. This selective histophysiological effect by 9-alpha-fluorohydrocortisone on the zona glomerulosa is more in keeping with clinical experience which has shown it to be equal to or more potent than aldosterone (14–16). These histological results conflict with certain physiological studies which demonstrated that 9-alpha-fluorohydrocortisone shows little sodium-retaining activity in the rat but produces marked sodium retention in other species such as dog and man (13).

It is interesting that 9-alpha-fluorohydrocortisone did not cause lipid depletion when given alone to the younger rats. However, the fact that it did cause lipid depletion of this zone when given in conjunction with ACTH is provocative. In 1955, Wexler and Rinfret (17), reported that, "certain, but not all species of ACTH will specifically deplete the lipid in the zona glomerulosa of the rat." On the basis of these histological observations they suggested that aldosterone and aldosterone-like steroids may be released under the influence of ACTH. Venning et al., (18) have recently shown that *in vitro* preparations of rat adrenal do produce increased amounts of aldosterone in response to certain ACTH preparations. It might well be that the older rats responded to the mineralocorticoid effects of 9-alpha-fluorohydrocortisone because of higher circulating levels of ACTH which "conditioned" the zona glomerulosa to the mineralocorticoid. On the other hand, younger rats having a lower titre of circulating ACTH could not respond to the 9-alpha-fluorohydrocortisone until exogenous ACTH was introduced. The well known pituitary ACTH-suppressing effects of 9-alpha-fluorohydrocortisone (19–21) may also have been operative.

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REFERENCES

1. GIROUD, C. J. P., J. STACHENKO AND E. H. VENNING: *Proc. Soc. Exp. Biol. & Med.* **92**: 154. 1956.
2. GREEP, R. O. AND H. W. DEANE: *Endocrinology* **40**: 417. 1947.
3. GOLDMAN, M. L., E. RONZONI AND H. A. SCHROEDER: *Endocrinology* **58**: 57. 1956.
4. DEANE, H. W. AND G. M. C. MASSON: *J. Clin. Endocrinol.* **11**: 193. 1951.
5. GREEP, R. O. AND H. W. DEANE: *Ann. N. Y. Acad. Sci.* **50**: 596. 1949.
6. GORDON, E. S.: *J. Lab. & Clin. Med.* **46**: 820. 1955.
7. VENNING, E. H., B. SINGER AND G. A. SIMPSON: *Am. J. Obst. & Gynec.* **67**: 542. 1954.
8. LEUTSCHER, J. A., JR. AND B. B. JOHNSON: *J. Clin. Invest.* **33**: 276. 1954.
9. CHART, J. J. AND E. S. SHIPLEY: *J. Clin. Invest.* **32**: 560. 1953.
10. GENEST, J., E. KOIW, W. NOWACZYNSKI AND G. LEBOEUL: *Canad. J. Biochem. & Physiol.* **35**: 425. 1957.
11. FRIED, J. AND E. F. SABO: *J. Amer. Chem. Soc.* **76**: 1455. 1954.
12. BORMAN, A. AND F. M. SINGER: *Fed. Proc.* **13**: 185. 1954.
13. FRIED, J.: *Ann. N. Y. Acad. Sci.* **61**: 573. 1955.
14. LIDDLE, G. W., M. M. PECHET AND F. L. BARTTER: *Science* **120**: 496. 1954.
15. RENOLD, A. E., N. A. HAYDAR, W. J. REDDY, A. GOLDFIEN, J. R. STMARC AND J. C. LAIDLAW: *Ann. N. Y. Acad. Sci.* **61**: 582. 1955.
16. THORN, G. W., R. H. SHEPPARD, W. I. MORSE, W. J. REDDY, P. M. BIEGELMAN AND A. E. RENOLD: *Ann. N. Y. Acad. Sci.* **61**: 609. 1955.
17. WEXLER, B. C. AND A. P. RINFRET: *Endocrinology* **57**: 608. 1955.
18. VENNING, E. H., O. J. LUCIS, I. DYREHFURTH AND J. C. BECK: Program of the 40th Annual Meeting of the Endocrine Society, San Francisco, California. 1958.
19. LIDDLE, G. W.: *J. Clin. Endocrinol. & Metab.* **16**: 557. 1956.
20. KUPPERMAN, H. S., M. H. G. BLATT, M. VESSELL, J. GAGLIANI, H. WEISBADER AND L. VOSBURGH: *J. Clin. Endocrinol. & Metab.* **15**: 911. 1955.
21. COPE, C. L. AND R. J. HARRISON: *Brit. M. J.* **2**: 457. 1955.

A MODIFICATION OF THE BATES-CORNFIELD BIO-ASSAY FOR THYROTROPIN[1,2]

ABSTRACT

Day-old chicks with low thyroidal uptake of radioiodine were used in the Bates-Cornfield bio-assay for thyrotropin. This was made possible by introducing an easily available correction factor for the circulating extrathyroidal radioactivity. It was concluded that the dose-response curves obtained by this method compare favorably to those obtained by the original method.

The method of Bates and Cornfield (1) is one of the few current methods that may be useful in the determination of thyrotropin (TSH) in human blood. This method is dependent on the use of chicks with a high uptake of I^{131}, and they are not easily available. A modification has therefore been worked out that permits the use of chicks with a low uptake of radioiodine.

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[1] Abridgment of portion of thesis submitted by Dr. Frey to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

[2] The radioisotopes used in the studies reported on in this paper were supplied by the United States Atomic Energy Commission.

MATERIAL AND METHODS

Plymouth White Rock chicks of mixed sexes were used. They were received on the day of hatching and were given I^{131} on arrival. They were kept at 35° C in brooders housing 50 chicks; they had free access to tap water but were starved routinely.

Solutions of propylthiouracil (PTU) and thyroxine were prepared as described by Bates and Cornfield (1). The daily dose per chick was 8 gamma thyroxine and 0.5 mg. propylthiouracil. The thyrotropin used was Armour's Lyophilized Thyrotropin, lot No. R 491075. Serial dilutions of thyrotropin were prepared immediately before use in 0.1% ovalbumin. The radioiodine used was carrier-free, pyrogen-free sodium iodide^{131}. The dose administered (1 to 5 μc.) was too small to cause any significant radiation damage to the thyroids of the chicks (Skanse [2]), and the birds used in the experiments have subsequently grown to be normal adults. The radioiodine was injected subcutaneously in the nape of the neck in a volume of 0.2 cc. Whole body counts immediately after injection on several occasions showed that the whole dose was retained. Injections of solutions of thyrotropin and of thyroxine also were done in the same way and at the same site.

A well-type scintillation crystal, connected to a scaler, was used for determination of radioactivity. Best results were obtained by using a lead collimator $\frac{1}{2}$ inch in thickness with a hole in the center 1 inch in diameter. The lead collimator was covered by a thin aluminum plate with a center mark. The chick was held in one hand, breast down, with the sternal notch above the center mark. Firm pressure maintained a constant distance to the crystal. The bird usually remained quiet during the procedure. The reproducibility of this counting method was $\pm 7\%$. On counting over the tail, the chick was pushed headwards as far as possible with the 1-inch hole still covered by the tail.

RESULTS

Figure 1 shows the effect of introducing the "tail count" as a correction factor for the extrathyroidal radioactivity in the neck (the "body background"). The difference

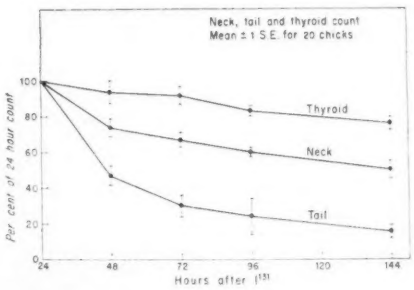

FIG. 1. Neck, tail and thyroid counts at various intervals after injection of I^{131}.

between the count obtained over the neck and the count obtained over the tail may be taken to represent the true thyroidal radioactivity, that is the "thyroid count." The relative constancy of the thyroid count will enable one to use this count as a baseline which is largely uninfluenced by changes in extrathyroidal radioactivity.

In Figure 1 the neck, the tail and the thyroid counts for each chick are expressed as percentages of the count for each category obtained 24 hours after the injection of I^{131}. Each point on the curve represents the mean value for 20 chicks. Propylthiouracil and thyroxine were administered in the usual doses every 24 hours, starting 24 hours after the injection of I^{131}.

It is apparent from Figure 1 that the curve for the thyroid count shows a slower fall than the curve for the neck and the tail counts. The 24% loss from the thyroid over a 5-day period conforms well with the loss of radioactivity from the blocked thyroid as determined by removal and direct counting of the glands (loss of 5% per day).

In trying to estimate how representative the tail count is for the extrathyroidal count in the neck, day-old chicks were given 0.5 mg. of propylthiouracil and 8 μg. of thyroxine 12 hours and again 1 hour before the injection of I^{131}. The effectiveness of propylthiouracil and thyroxine in preventing uptake of radioiodine by the thyroid was demonstrated by

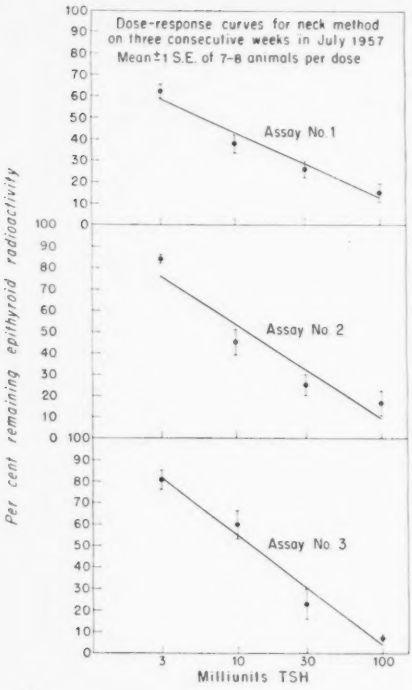

FIG. 2. Three dose-response curves for thyrotropin.

killing some of the birds 24 hours after the injection of I^{131} and determining the uptake of I^{131} by the thyroids. The glands showed a negligible uptake of I^{131}. Counts were made over the neck and the tail 3, 6, 12, 24, 48, 72 and 92 hours after the injection of I^{131}. The counts showed a parallel fall with a ratio of tail count to neck count varying slightly around 0.80. This indicated that the tail count at all times represented an 80% correction for the extrathyroidal radioactivity in the neck.[3]

The influence of the backscatter from the thyroid on the tail count was found negligible, as proved by experiments using varying doses of I^{131} applied externally with adhesive tape over the thyroid.

The tail count was found to be an easily available and fairly accurate correction factor for the body background, and assays were carried out by means of the technic of Bates and Cornfield (1). The chicks arrived in the evening and were given I^{131} on arrival; the first reading was obtained in the morning 36 hours later. This reading served as the 100% value for each chick. The chicks were not selected according to body weight or thyroid uptake but they were picked at random with 7 to 8 chicks per dose. After the initial reading, thyrotropin was given in divided doses, one injection daily for 3 days. On the fourth day only a count was done; it was expressed in per cent of the initial reading after correction for physical decay of the isotope. Figure 2 shows the dose-response curves obtained on three consecutive weeks in July, 1957. Each point represents the mean thyroid count for each dose level. Figure 2 gives the regression lines, slope and index of accuracy for the three assays.

COMMENT

The three curves differ slightly in their characteristics. This is partly artificial, because the curves reach an asymptote before the dose of 100 milliunits (U.S.P.) of thyrotropin. Therefore, when the dose of 100 milliunits is included in the computation of the regression line, the slope will be artificially lowered. This factor is especially prominent in assay No. 1. For the three assays, the slopes were −30, −44 and −51; the λ values were 0.36, 0.26 and 0.20, respectively.

[3] As was pointed out in the text, the tail count represents the general level of I^{131} in the tissue. However, a few chicks have a disproportionately high tail count on the initial reading, probably due to accumulation of radioactive material in the excretory organs. These animals were excluded for assay purposes if the ratio of neck count to tail count on the initial reading was less than 3:1. About 10% of the animals do not meet this requirement.

Only minor variations in animal sensitivity were encountered from week to week in July. However, at least a tenfold variation in animal responsiveness occurs. Thus in the late fall months the extinction-point was higher than 10 milliunits, while in February, 1958, it was 0.3 milliunit. The curves presented compare favorably with the original method of Bates and Cornfield.[4] Sensitivity, accuracy and range are almost identical. The slope may be higher in the original method, but, considering the artificially lowered slope in the curves presented here, this difference is not so great as to be of practical importance.

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REFERENCES

1. BATES, R. W. and JEROME CORNFIELD: *Endocrinology* **60**: 225. 1957.
2. SKANSE, B. N.: *J. Clin. Endocrinol.* **8**: 707. 1948.

[4] The original method of Bates and Cornfield requires chicks with an average thyroid uptake of radioiodine of at least 20 per cent of the dose. The chicks used in the present study had an average 24-hour uptake from May to October of 8%. It is obvious that in chicks with a low uptake the extrathyroidal radioactivity in the neck will influence the neck count to a disturbing degree. But this also may hold true for chicks with a high uptake. Thus we did not find any correlation between the radioactivity in the thyroid and the radioactivity in the rest of the chick (called the "carcass") when counts were made separately 24 hours after the injection of I^{131}. Even chicks with a relatively high thyroidal uptake (in excess of 20%) might have retained more than 40% of the dose in the carcass. Therefore, the extrathyroidal radioactivity may have to be taken into consideration even in a chick with high thyroidal uptake.

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AN EFFECT OF VASOPRESSIN ON RENAL WEIGHT

ABSTRACT

It has been found that the administration of "Pitressin" or synthetic lysine vasopressin will bring about an increase of the weight of kidneys of hypophysectomized rats. An effect was noted with as little as 10 milliunits of synthetic lysine vasopressin. Normal rats and operated control or saline injected hypophysectomized rats did not respond with a change in renal weight.

Male Sprague-Dawley rats about 6 months in age were used in the study. The hypophysectomized rats weighed 100–150 gm. and were three months postoperative with no observed growth response. The hormone preparations were "Pitressin" (Parke, Davis) and synthetic lysine vasopressin (LVS_2) supplied by Dr. Vincent du Vigneaud. The animals were anesthetized with ether (identical results were obtained with chloralose), one kidney was removed and the hormone preparation was administered in the tail vein in 0.1–0.3 ml. saline. After one hour, the remaining kidney was removed, the organs were freed of connective tissue, blotted on a filter paper and weighed.

RESULTS AND DISCUSSION

The increases of kidney weight versus dose of administered hormone are shown in Figure 1; six animals were used for each of three of the points. The average deviation

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was less than 4%. At the dosage of 60 milliunits (the third point) 16 hypophysectomized rats were tested. At this level the average increase was 13.3% and the average deviation from this average was ±4.6%. In a group of 9 normal rats of the same strain and age receiving 600 milliunits of vasopressin the increase was 0.3±3.6%; in a group of 6 saline-injected hypophysectomized rats, the change of renal weight was −1.2±2.8%. At higher levels of vasopression (2000 and 3000 milliunits) the increase in renal weight at one hour approximated 20%; it was not possible to distinguish between these two levels. In the lower dosage levels, the difference between 6 and 12 milliunits was not easily distinguishable with the small samples. In all 35 control animals, the average change in renal weight was 3.2%.

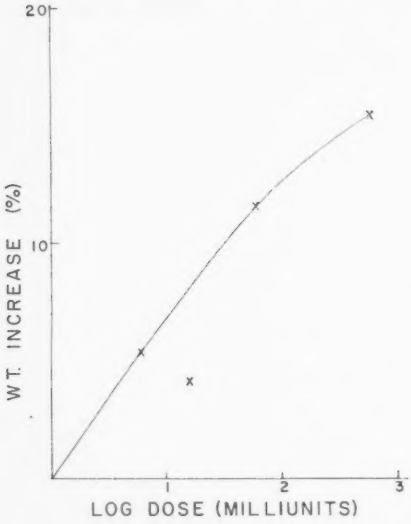

FIG. 1. Increase in kidney weight plotted against log dose (in milliunits) of synthetic lysine vasopressin-LVS2.

Some studies were made of the maximally stimulated kidneys (2 units vasopressin); the kidneys were homogenized in water and the content of hemoglobin (as oxyhemoglobin) and of protein (1) was determined. There were no differences noted in the analyses of 6 pairs of kidneys and one must conclude that water was the principal component responsible for the gain in weight. The effect would seem to be quite specific; a variety of proteins and protein hydrolysates injected in amounts of 0.1 to 1.0 mg. had no effect on renal weight. It would appear that the effect is sensitive and specific enough to serve as a test for moderate amounts of vasopressin. The time relationship has not been investigated in detail but easily detectable differences were noted with 60 milliunits at 15 minutes and the effects at 30 and 60 minutes were nearly identical.

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REFERENCE

1. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR AND R. S. RANDALL, *J. Biol. Chem.* **193**: 265. 1951.

[1] We are indebted to Frances Jobson for technical assistance.

OXYTOCIN AND OXYTOCIC SUBSTANCE IN BLOOD EXTRACTS BEFORE, AND FOLLOWING, COPULATION IN SHEEP

ABSTRACT

Ewes in natural oestrus showed no increase in the concentration of oxytocin in blood extracts following stimulation by copulation.

There is indirect evidence that, during natural and artificial insemination, oxytocin is released into the blood from the hypothalamic-posterior-pituitary complex (1). Milk ejection in lactating mothers has been frequently observed in association with coitus, and it has been postulated in animals that the powerful uterine contractions induced by the stimulus of copulation may assist in the rapid transit of spermatozoa into the fallopian tubes.

To our knowledge no one has actually measured oxytocin in the blood in these circumstances, so it was decided to investigate the effect of copulation on the blood level of oxytocin in sheep.

METHOD

Samples of jugular blood (approx. 10 ml.) were taken from 7 ewes (in natural oestrus) before, and immediately after, copulation. The blood specimens were then extracted and assayed for oxytocic activity, oxytocin and oxytocic substance as previously described (2).

RESULTS AND DISCUSSION

Table 1 shows that in 7 ewes the total oxytocic activity of the blood extracts decreased in 6 and increased in 1. The changes observed in ewes 6 and 7 were of doubtful significance, and if these are exluded then a fall in total oxytocic activity was registered

TABLE 1. OXYTOCIC ACTIVITY OF ACID-ALCOHOL EXTRACTS OF BLOOD (SHEEP) BEFORE, AND AFTER, COPULATION

No.	Oxytocin	Tot. activity	Oxytocic substance	Tot. activity	Percentage change in total activity	Percentage change in oxytocin	Percentage change in oxytocic substance	Oxytocin		Oxytocic substance	
	Percentage		Percentage					Mu./ml. blood			
	Before	After	Before	After				Before	After	Before	After
1					−88.9	—	—	2.24		1.2	
2					−32.6	—	—	—		—	
3	0	0	100	100	−35.3	0	−11.8	0	0	0.17	0.15
4	0	0	100	100	−20.0	0	−20.0	0	0	0.25	0.20
5	0	0	100	100	−16.6	0	+10.3	0	0	0.29	0.32
6	0	0	100	100	+11.1	0	0	0	0	0.45	0.45
7	1.25	20.6	98.75	79.4	− 7.5	1500	−26.6	0.01	0.16	0.79	0.58

in all cases. In 5 ewes from whom the blood extracts were assayed for oxytocin and oxytocic substance, the level of oxytocin was unchanged in 4 and increased in 1, whilst the concentration of oxytocic substance did not significantly increase in any.

It is interesting to note that, in these experiments on sheep, there is virtually no oxytocin detectable in the blood extracts. In this respect sheep differ considerably from rats (3) since in the latter the concentration of oxytocin is invariably in the order of several MU./ml. blood. In sheep the oxytocic activity of blood is almost entirely due to the presence in the extracts of oxytocic substance. It is now thought that the latter is a polypeptide, and that it is not to be confused with such other oxytocic factors as oxytocin, potassium, acetylcholine, 5-hydroxytryptamine, hypertensin, vasopressin, pepsitensin, pepsitocin, pepsanurin, substance P, pain-producing substance or kinin (bradykinin, kallidin, substance U or substance Z).

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REFERENCES

1. VAN DYKE, H. B., K. ADAMSONS AND S. L. ENGEL: *Rec. Prog. Horm. Res.* **11**: 38. 1955.
2. HAWKER, R. W. AND P. A. ROBERTSON: *Endocrinology* **60**: 652. 1957.
3. BISSETT, G. W. AND J. M. WALKER: *J. Physiol.* **126**: 588. 1954.

COMPARISON OF *IN VITRO* AND *IN VIVO* ASSAYING PROCEDURES FOR RAT ADENOHYPOPHYSIAL CORTICOTROPHIN[1]

ABSTRACT

The same rat pituitary extract was concurrently assayed against the same standard (U.S.P. Reference Standard Corticotropin) by procedures respectively based on *in vitro* corticoidogenesis, and on *in vivo* corticoidogenesis and adrenal ascorbic acid depletion. Nearly identical values (20–24 mU. ACTH/mg. fresh tissue) were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration (62 mU./mg.) was recorded on the basis of *in vitro* corticoidogenesis. These findings do not support the hypothesis of Birmingham *et al.* of a higher *steroidogenic/ascorbic acid-depleting* activity ratio in rat pituitary extract than in the reference standard. The discrepancy in the results yielded by the two types of procedures appears to be related to differences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or stimulation or corticoidogenesis) of the assays.

Consistently higher values for pituitary corticotrophin concentration in the intact rat have been obtained with Saffran and Schally's *in vitro* corticoidogenic assay (1) than with Sayers' *in vivo* adrenal ascorbic acid depletion method (2). Due allowances being made for the different strains of rats and extraction procedures used by various groups, the values recorded, in terms of milliunits (mU.) of ACTH per mg. of fresh tissue, range from 10 to 38 (3–6) with Sayers', and from 52 to 241 (7, 8) with Saffran and Schally's assay. A mean concentration of 64.6 ± 1.8 mU./mg. was recorded by one of us (9) from one hundred *in vitro* assays (average λ:0.106 ± 0.004) of 0.1N HCl-extracted pituitaries from intact male Sprague-Dawley rats.[2]

To account for the discrepancy in the results yielded by the two assaying procedures, Birmingham *et al.* (7) suggested that the *steroidogenic/ascorbic acid-depleting* (S/AA) activity-ratio might be higher in native (pituitary extract) than in more purified (Reference Standard) corticotrophin. The following experiment was aimed at testing this possibility by concurrently assaying the same rat pituitary extract against the same standards, through procedures respectively based on *in vitro* corticoidogenesis, and on *in vivo* corticoidogenesis and adrenal ascorbic acid depletion.

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[2] Obtained from Holtzman Rat Company.

One day prior to assay, the adenohypophyses from two adult male Sprague-Dawley rats, killed by decapitation, were quickly removed from the skull, separated from the neural lobe, wiped clean of blood, weighed to the nearest 0.05 mg., pooled in a 15 ml. conical centrifuge tube containing 0.4 ml. of 0.1N HCl, and ground with a glass rod and a few grains of sand. The rod was rinsed into the tube with an identical volume of 0.1N HCl, and the stoppered extract, stored in the deepfreeze. A weighed sample of U.S.P. Reference Standard Corticotropin dissolved in 0.1N HCl was stored at the same time. On following morning, extract and standard were thawed, adjusted to 0.01 normality, and left for two hours at room temperature. A four-point-two-dose ($D^1/D^2=\frac{1}{2}$) design was used for *in vitro* and *in vivo* assays. On the basis of previous experiments, the S^2 solutions of the standard were respectively adjusted to 100 mU. ACTH/ml. for the *in vitro*, and to 5 mU./ml. for the *in vivo* assays; the U^2 solutions of the extract, to 1.9 mg. pituitary/ml. for the *in vitro*, and to 0.22 mg./ml. for the *in vivo* assays. Aliquots of 0.3 ml./100 mg. adrenal were used in the *in vitro*, of 0.3 ml./100 gm. B.W., in the *in vivo* assays.

The *in vitro* assays were performed by the technique of Saffran and Schally (1). The *in vivo* assays were based on the a) adrenal ascorbic acid, b) adrenal corticosteroid, and c) plasma free-corticosteroid responses of 24-hour hypophysectomized male rats (Hormone Assay Laboratories, Chicago; 6 rats per dose) to the injection of extract or standard. The solutions were injected in a randomized fashion, by the intrajugular route, under ether anesthesia. 15 minutes later,[3] blood was collected with a heparinized syringe from the abdominal aorta for determination of the plasma free corticosteroids by the fluorometric method of Silber, as modified by Guillemin *et al.* (10), and both adrenals were removed, dissected free of fat, and weighed to the nearest 0.1 mg. The left gland was extracted in 4% trichloracetic acid for ascorbic acid determination by a modification (solutions incubated for 15 minutes (vs. 180) at 100° C (vs. 37° C) of Roe and Kuether's method (11). The right gland was extracted in alcoholic saline prior to corticosteroid determination by fluorometry (12). The results, computed and tested for validity according to Bliss' methods for factorial analysis and analysis of variance (13), were expressed in mU. of ACTH per mg. of fresh tissue, with confidence limits (antilog $C^2M \pm CtSm$) for odds of 19 in 20. Equal emphasis was placed on the internal (*within* assays) and external (*between* assays) variances of the combined estimates (unweighted logarithmic means) by computing their standard errors from the averages of the crude and unweighted Sm's (13).

TABLE 1. COMPARATIVE ASSAYS OF THE SAME RAT PITUITARY EXTRACT AGAINST THE SAME STANDARD BY *in vitro* AND *in vivo* TECHNIQUES

Type of assay	ACTH per mg. fresh tissue (mU.)	95% confidence limits (mU.)	Index of precision (λ)
In Vitro Corticoidogenesis			
#1	69.5	45.8–116.6	0.080
#2	42.5	23.5– 69.2	0.104
#3	60.8	46.8– 80.3	0.057
#4	80.6	40.2–314.7	0.157
Mean ± S.E.	61.7 ± 7.2		0.099 ± 0.026
In Vivo			
A. Adrenal Ascorbic Acid	20.8	15.8– 27.1	0.081
B. Adrenal Corticosteroids	20.1	18.3– 22.2	0.028
C. Plasma Free Corticosteroids	24.4	20.4– 27.1	0.034
Mean ± S.E.	21.7 ± 1.2		0.048 ± 0.017

RESULTS AND CONCLUSION

Nearly identical values were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration was again recorded on the basis of *in vitro* corticoidogenesis (Table 1). These findings do not support Birmingham *et al.'s* hypothesis of a higher S/AA activity ratio in rat pituitary extract than in the reference standard. The discrepancy would thus appear to be related to differ-

[3] This interval was observed to correspond to maximal adrenal ascorbic acid and corticosteroid responses to ACTH (10).

ences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or stimulation of corticoidogenesis) of the assays. Further studies aimed at elucidating this problem are reported elsewhere (9).

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REFERENCES

1. SAFFRAN, M. AND A. V. SCHALLY: *Endocrinology* **56**: 523. 1955.
2. SAYERS, M. A., G. SAYERS AND L. A. WOODBURY: *Endocrinology* **42**: 379. 1948.
3. BURNS, T. W., M. MERKIN, M. A. SAYERS AND G. SAYERS: *Endocrinology* **44**: 439. 1949.
4. GEMZELL, C. A., D. C. VAN DYKE, C. A. TOBIAS AND H. M. EVANS: *Endocrinology* **49**: 325. 1951.
5. GEMZELL, C. A.: *Endocrinology* **55**: 621. 1954.
6. SYDNOR, K. L. AND G. SAYERS: *Endocrinology* **55**: 621. 1954.
7. BIRMINGHAM, M. K., E. KURLENTS, G. J. ROCHEFORT, M. SAFFRAN AND A. V. SCHALLY: *Endocrinology* **59**: 677. 1956.
8. KITAY, J. I., D. A. HOLUB AND J. W. JAILER: *Proc. Soc. Exp. Biol. and Med.* **97**: 165. 1958.
9. FORTIER, C.: *Proc. Soc. Exp. Biol. and Med.* To be published, December 1958.
10. GUILLEMIN, R., G. W. CLAYTON, J. D. SMITH AND H. S. LIPSCOMB: *Endocrinology* **63**: 349. 1958.
11. ROE, J. H. AND C. A. KUETHER: *J. Biol. Chem.* **147**: 399. 1943.
12. GUILLEMIN, R., G. W. CLAYTON, H. S. LIPSCOMB AND J. D. SMITH: *J. Lab. and Clin. Med.* To be published, May 1959.
13. BLISS, C. I.: *The Statistics of Bioassay.* New York: Acad. Press. 1952.

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MODIFICATION OF THE CATABOLIC ACTION OF ACTH IN RATS FED A DIET HIGH IN POTASSIUM[1]

ABSTRACT

Groups of normal female rats were maintained on a constant food intake and daily urine collections were made for nitrogen analysis. Experimental animals received an extra 600 mg. per day of KCl in their diet. ACTH assaying 15 USP units per mg. was given in doses ranging from 10 μg. to 200 μg. per rat per day. At doses of ACTH below 100 μg. per rat per day no significant increase in urinary nitrogen occurred in those animals on the high KCl diet whereas it did occur in animals on the control diet. With doses of ACTH of 100 μg. or above per rat per day it occurred in both groups, but was more marked in the control animals.

In a previous paper from this laboratory (1) it was reported that the inclusion daily of an extra 600 mg. of KCl in the diet of normal female rats inhibited the catabolic effect of administered ACTH. In these experiments ACTH administration was started

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at such low dosage that no catabolic effect was observed either in the control animals or in the animals on the high KCl intake. As the dose of ACTH was increased, a definite catabolic effect appeared in the control animals. At this time no group of animals on a high KCl intake showed a statistically significant increase in nitrogen excretion, although some groups of animals on the high KCl intake did show small increases in urinary nitrogen. Subsequently, Rupp, Paschkis, and Cantarow (2), using tube fed animals receiving amounts of extra KCl identical to those employed in the experiments reported by this laboratory were unable to demonstrate an inhibition of the catabolic effect of either ACTH or cortisone. Their experiments differed, however, from those reported from this laboratory in that they used only one dose level, and a fairly large one, of both ACTH and cortisone. The dose of cortisone which they employed was 5 mg. per rat per day and they used 10 units of ACTH per rat per day. We have carried out experiments in an effort to repeat the original observation of Whitney, Bennett, and Li (1). Also, these experiments have been designed to determine whether if one used progessively larger doses of ACTH, its catabolic effect would reappear even though a diet high in KCl were being fed.

METHOD

The animals used, the diet, the methods of collection of urine and of analyses, the control of food intake and the amount of extra KCl added to the diet was identical with those previously reported (1). There were five animals in each group of animals in each experiment. Urine collections were started after a period of a week or more in the metabolism cages. The ACTH used was the ACTH preparation E obtained by elution from oxycellulose (3). The preparation assayed 15 USP units per mg. and was administered subcutaneously in bees wax.

RESULTS AND COMMENTS

The results of the experiments in which ACTH was administered are reported in Table 1. It will be noted that in three different experiments in which 40 μg. of ACTH was given per day a significant increase in urinary nitrogen occurred in the groups on the control diet on two occasions, whereas no significant increase occurred in any group on the high KCl diet. When the dose of ACTH was doubled to 80 μg. per day a significant increase in urinary nitrogen occurred in one of the two control groups but in neither of the groups on the high KCl diet. In four experiments when the dose of ACTH was 100 μg. or more per day significant increases in urinary nitrogen occurred in all control groups and in one of the groups on the high KCl intake. Also it is apparent from the table that in seven of nine periods there was an increase in urinary nitrogen when ACTH was given to animals on the high KCl diet. However, in each of these instances the increase in urinary nitrogen of the animals on the control diet was greater than that

TABLE 1. CHANGE IN URINARY NITROGEN OF ACTH TREATED ANIMALS

Experiment		Average urinary nitrogen during control period:* mg./day	Average change in urinary nitrogen during ACTH treatment†: mg./day									
			40 μg. ACTH /day	p	80 μg. ACTH /day	p	100 μg. ACTH /day	p	100 μg. ACTH /day	p	200 μg. ACTH /day	p
15D	Control	402 ± 3.7	+37	.03								
	High KCl	400 ± 2.6	+13	.10								
15E	Control	428 ± 7.3	− 4	>.80	+21	.10			+29	.05		
	High KCl	435 ± 4.7	−12	>.80	−17	>.80			− 9	>.90		
15B	Control	323 ± 4.6	+40	<.01	+47	<.01			+67	<.01		
	High KCl	344 ± 7.1	+ 6	.60	+12	.50			+24	.10		
15A	Control	348 ± 4.9									+55	<.001
	High KCl	336 ± 4.9									+26	.10
15F	Control	297 ± 4.5					+110	<.001				
	High KCl	291 ± 4.9					+ 47	.03				

* The control period was six or seven days in duration.
† The treatment period was three or four days in duration.

of the animals on the high KCl diet. With increasing doses of ACTH its catabolic effect is more marked both in the control animals and in those on the high KCl intake. Thus, the average change in urinary nitrogen of control animals receiving less than 100 μg. of ACTH per day was an increase of 28 mg. per rat per day; whereas, the average change in high KCl animals at the same dosage was less than 1 mg. per rat per day. At doses of 100 μg. or more per day of ACTH the average increases in urinary nitrogen were 65 mg. per rat per day for those on the control diet and 28 mg. per rat per day for those on the high KCl diet.

In confirmation of previous results (1) there was no demonstrable difference in adrenal size when the weights of the adrenals of the ACTH treated animals on the control diet and the adrenals of the ACTH treated animals on the high KCl intake were compared. Likewise, when these adrenals were studied microscopically after staining by the Flexner Grollman technique, (4) no differences between the two groups were found.

It is worthy of note that in these experiments at daily doses of ACTH below 1.5 units per rat per day, no significant increases in urinary nitrogen occurred in animals on the high potassium diet. At doses of 1.5 units or more per rat per day significant increases in urinary nitrogen occurred in both groups. As pointed out earlier, Rupp, Paschkis, and Cantarow used only doses larger than these in their experiments in which they reported failure to confirm the results of Whitney and Bennett.

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REFERENCES

1. WHITNEY, J. E. AND L. L. BENNETT, *Endocrinology* **50**: 657. 1952.
2. RUPP, J. J., K. E. PASCHKIS AND A. CANTAROW, *Endocrinology* **56**: 21. 1955.
3. LI, C. H., *Acta Endocrinol.* **10**: 255. 1952.
4. FLEXNER, L. B. AND A. GROLLMAN, *Anat. Record* **75**: 207. 1939.

A TECHNIQUE FOR REMOVING THYROID GLANDS FROM ANURAN LARVAE[1]

ABSTRACT

It is pointed out that removal of thyroidal primordia from embryos is unsatisfactory as a means of obtaining thyroidectomized larvae since the gland often regenerates. A technique for removing the gland from the tadpole is described and cited as producing uniformly good results.

Thyroidectomized tadpoles are occasionally needed in large numbers for endocrinological investigations. Allen (1) has described a technique for removing thyroid anlagen from embryos at tailbud stages of development, but his technique is rather tedious and time consuming. Also, Dr. William Etkin (personal communication) reports that he, as well as other workers, find that the gland regenerates in at least 50% of animals operated upon in the tailbud stage even when the entire floor of the pharynx is removed. The technique to be described was cited earlier (2). It has been used in the successful extirpa-

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tion of thyroid glands from *Rana pipiens, R. palustris, R. catesbeiana, Bufo americanus,* and *Hyla versicolor* at all stages of larval development; but the operation was most conveniently carried out on animals at the stage during which yolk consumption is being terminated and feeding is beginning to take place. At that stage, the thyroid still contains yolk granules and is easily distinguished from surrounding tissues so that complete removal of the gland is readily accomplished. Furthermore, younger larvae seem to be less adversely affected by operational trauma than older animals.

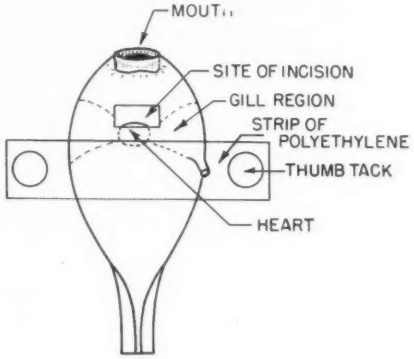

FIG. 1

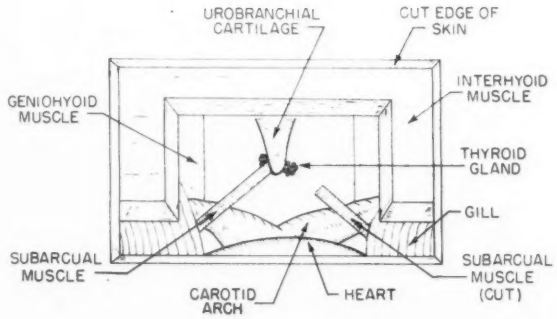

FIG. 2

In preparation for the operation, the tadpole is anesthetized in tricaine methanesulfonate (MS-222, M. Sandoz, New York) at a concentration of 1:3000 in spring water. The animal is then placed on its back within a depression molded in the bottom of a wax-lined operating dish. It is secured by means of a strip of flexible plastic material (polyethylene sheeting) which, in turn, is held in place with thumbtacks (Fig. 1). Cutting is done under a dissecting microscope by inserting a knife edge ground on the tip of a dissecting needle beneath the tissue to be cut and then working the tips of a pair of watchmaker's forceps against the knife edge through the tissue. First, a rectangular piece of skin is removed just anterior to the pericardial cavity, exposing the medial portion of the interhyoid muscle. The interhyoid is a relatively broad, flat muscle with fibers running transversely (Fig. 2). The posterior half of this medial portion (or the complete medial portion in younger animals) of the interhyoid is then removed. Through the

opening thus created the urobranchial cartilage is seen extending ventrocaudad from the hypobranchial plate of the hyoid apparatus. A pair of subarcual muscles are attached to the urobranchial cartilage and run obliquely caudolaterad from it. When these subarcual muscles are transected, the lobes of the thyroid are seen lying against the hypobranchial plate lateral to the urobranchial cartilage, medial to the geniohyoid muscles, and craniodorsad to the carotid arches. Finally, the exposed thyroid lobes are removed by aspiration through a capillary pipette with a mouth-controlled suction tube attached to it.

Tadpoles operated on in this fashion required no special postoperative care. They were merely put in ordinary culture dishes or aquaria, where rapid healing ensued. Removal of the thyroid was demonstrated to be complete, and mortality was negligible.

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REFERENCES

1. ALLEN, B. M. *Science* **44**: 755. 1916.
2. DENT, J. N. AND E. L. HUNT: *J. Exptl. Zool.* **121**: 79. 1952.

[2] Operated by Union Carbide Corporation for the United States Atomic Energy Commission. (Work done under subcontract No. 701)

THE USE OF THE TOAD FOR BIOASSAY OF MAMMALIAN ANTIDIURETIC HORMONE (VASOPRESSIN)[1]

ABSTRACT

Injection of mammalian antidiuretic hormone (ADH) into toads produced increased water uptake and decreased urine production, resulting in weight gain over the pre-injection period. A bioassay for ADH, based on this response, has been developed for *Bufo marinus*, a large toad available in the United States. This method is an adaptation and modification of an assay devised by Buchborn for the European toad *Bufo* bufo. Details of the assay procedure are given, as well as instructions for maintaining the toads in the laboratory.

The role of the neurohypophyseal hormone, vasopressin, in body water and electrolyte balance has long been of interest to both physiologists and clinicians. The determination of circulating levels of this hormone has, however, been hampered by the capricious nature and limited sensitivity of the assay methods which have been available. Most of these methods depend on the excretion of an administered water load by rats or other mammals. But pain, trauma, stress, anesthesia, and even noise, temperature changes or strange environment may interrupt the expected diuresis in a water loaded animal, presumably by effecting an increase in endogenous hormone production.

Brunn (1) first pointed out that amphibians respond to mammalian neurohypophyseal extracts by increased water uptake through the skin. Many subsequent workers

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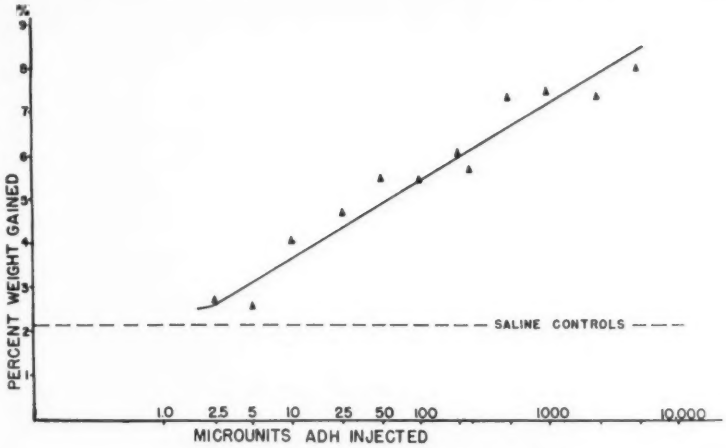

FIG. 1. Dose-response curve for *Bufo marinus* given various dosages of Pitressin[2] (3). The abscissa is a logarithmic scale, one microunit being one millionth of an International (pressor) Unit. Each triangular point represents the mean response of five toads at the third hour after injection. The dashed line represents the average weight gain, after the same interval, of toads injected with saline only. (The zero point on the ordinate scale represents the mean hydrated weight, or pre-injection weight, of each toad; since the injected fluid constitutes five percent of the animal's body weight, every animal will be heavier at the next weighing. However, this net gain is gradually lost by animals who receive only saline; it is maintained or increased under the influence of ADH).

have confirmed this fact, and have further found that toads are more sensitive than frogs to vasopressin. Buchborn (2) utilized this sensitivity in developing an assay method for antidiuretic hormone (ADH) using the European sand toad *Bufo bufo*. This author claims his method to be specific and sensitive from 10 μU. to 1500 μU. ADH.

We have adapted and modified this assay procedure for use with toads available in this country. The giant toad, *Bufo marinus*, has been used in the present study. This species is widespread in Central and South America, and its range extends northward into the southern tip of Texas. The animals are easily obtained because several supply houses stock them for pregnancy tests.

METHOD

For each assay, five male toads are hydrated by allowing them to remain in water overnight. The mean hydrated weight of each toad is then determined from three hourly weight determinations. After each weighing, the animal is caused to excrete his accumulated urine by catheterization with a blunt pipette, combined with slight pressure on the lower abdomen. Thus each weight determination represents base weight plus water taken up during the previous hour. Premature micturation with large variations in weight has not proved a problem. Each animal is then given an injection—5 cc. for every 100 gm. body weight—of vasopressin in saline,[2] into the dorsal lymph sac. Within two to five hours, depending on the size of the toad, a maximum weight gain (ADH) or return to original pre-injection weight (saline controls) is observed. For *B. marinus* weighing 80–120 gm., the mean response of five toads at three hours after injection is taken as the end point.

The magnitude of the response is directly proportional to the dosage of hormone injected (Fig. 1). When response of the toads (per cent weight gain) is plotted against log-dose, the curve follows a straight course from less than 5 μU. to 5,000 μU. (5 mU.).

[2] Pitressin, mfg. by Parke, Davis & Co., Detroit, Mich.

The toads are force-fed chopped raw beef liver once a week. During the period of this study (January-June), they appeared to remain healthy and many gained weight on this regime. No seasonal variations in response of the toads to hydration or hormone injection were noted during this period.

The present assay procedure has been used to determine levels of antidiuretic substance in rat plasma under a variety of conditions.[3] Preliminary studies have been conducted on human plasma. Work is in progress on dose-response curves for several other species of North American toads, in the hope of finding another species which may be even more senstivie to vasopressin, or more readily obtainable, or both.

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REFERENCES

1. BRUNN, F.: *Z. ges. exp. med.* **25**: 170. 1921.
2. BUCHBORN, E.: *Z. exper. med.* **125**: 614. 1955.

In the December 1958 issue of ENDOCRINOLOGY four manuscripts listed below should bear date of receipt as follows:

[3] A full account of this work will be published elsewhere.

CORRECTIONS

In the December 1958 issue of ENDOCRINOLOGY four manuscripts listed below should bear date of receipt as follows:

Bernstein, Donald E.
Autotransplantation of the Adrenal of the Rat to the Portal Circulation: Effect of Administration of Sex Hormones, 765. Received May 6, 1958

Evans, Edward S., Miriam E. Simpson and Herbert M. Evans
The Role of Growth Hormone in Calorigenesis and Thyroid Function, 836. Received June 2, 1958

Yates, Eugene F., Arthur L. Herbst and John Urquhart
Sex Difference in Rate of Ring A Reduction of Δ^4-3-Keto-Steroids *In vitro* by Rat Liver, 887. Received June 12, 1958

Moltke, Erik
Effect of Thyroxine and a Dehalogenase-Inhibitor on the Tensile Strength of Healing Wounds, 931. Received June 6, 1958

THE AMERICAN SOCIETY FOR THE STUDY OF STERILITY

The Fifteenth Annual Meeting of The American Society for The Study of Sterility will be held at The Shelburne Hotel, Atlantic City, N.J., April 3–5, 1959. Persons interested in attending this meeting should apply for advance registration and a complete program to the Secretary, Dr. Herbert H. Thomas, 920 South 19th Street, Birmingham 5, Alabama.